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Doctor of Philosophy degree in Food and Human Health

Cycle XXXI

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"Development of innovative extraction and detection methods for gluten based on deep eutectic solvents and aptamers"

Ph. D. candidate

Rossella Svigelj

Supervisor

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Nothing in life is to be feared, it is only to be understood.

Marie Skłodowska-Curie

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Abstract

Celiac disease is an autoimmune disease, affecting about 1% of the global population which is triggered by gluten. Currently the only effective therapy available is a strict gluten-free diet. Hence, people affected by celiac disease can eat naturally gluten-free products or food specifically produced gluten-free. The term “gluten” refers to the entire protein component of wheat, while gliadin is the alcohol-soluble fraction of gluten that contains a bulk of the toxic components.

These kinds of dietetic foods are regulated by the “Codex Alimentarius standard for foods for special dietary use for persons intolerant to gluten”, that sets a limit value of 20 mg/kg of gluten in these products.

The aim of this thesis was the development of an analytical method for the reliable detection of gliadin in food. Regardless of all the advances that have occurred so far, the development of gluten quantification methods still encounters significant difficulties derived from the identity of the allergen, the lack of an effective and universal extraction method and the availability of few receptors of sufficient affinity and selectivity in order to obtain a reliable analytical method. On the other hand, considering that the use of deep eutectic solvents (DESs) as extractants is an expanding field as well as the use of aptamers as recognition elements. We combined these two aspects to bring advances in gluten quantification in food. For this purpose, the suitability of two DESs, ethaline and reline, to extract gliadin (50% content of gluten) was evaluated employing a commercially available ELISA kit. Moreover, we performed the selection and characterization of new ssDNA aptamers as biological receptors for gliadin in DESs. We used SELEX, which is a universal and iterative process, where an initial degenerated library of oligonucleotides is challenged against the analyte, to select sequences with the highest affinity. We took a step further by demonstrating the viability of SELEX in a green extraction solvent for the first time, thus providing a method for obtaining aptamers able to recognize non-soluble and poorly water-soluble molecules or species prone to aggregation in aqueous solutions. Lastly, we developed a competitive assay sufficiently sensitive and selective to be applied to the determination of gluten in foods labeled "gluten-free". The competitive assay confirmed the usefulness of the selected aptamer for the direct detection of gluten in ethaline extracts, without extra-dilutions. It is important to highlight that this approach allows gluten extraction and its quantification in ethaline without any dilution of the sample. Therefore, it allows to reach very low quantification limits.

Index

Abbreviations

HLA	Human Leukocyte Antigen
AACCI	American Association of Cereal Chemists International
AOAC	Association for Official Analytical Chemists
AOACI	Association of Official Analytical Chemists International
BSA	Bovine Serum Albumin
CD	Celiac Disease
CE	Capillary Electrophoresis
ChCl	Choline Chloride
DESs	Deep Eutectic Solvent
DPV	Differential Pulse Voltammetry
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assays
FDA	Food and Drug Administration
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HRP	Horseradish Peroxidase
HTS	High-Throughput Sequencing
ILs	Ionic Liquids
ITC	Isothermal Titration Calorimetry
LFD	Lateral Flow Device
mAb	Monoclonal Antibody
MPs	Magnetic Particles
MST	Microscale Thermophoresis
MW	Molecular Weight
MWCNT–SPEs	Multiwalled Carbon Nanotube-modified Screen-Printed Electrode
NADES	Natural Deep Eutectic Solvents
NHS	N-hydroxysuccinimide
o-ABA	o-aminobenzoic acid
pAb	Polyclonal Antibody
PABA	Poly o-aminobenzoic acid
PBS	Phosphate Buffered Saline
PC SW	Photonic Crystal Surface Wave
PCR	Polymerase Chain Reaction
PSA	Prostate Specific Antigen
PWG	Prolamin Working Group
QCM	Quartz Crystal Microbalance
RSD	Relative Standard Deviation
SELEX	Systematic Evolution of Ligands by EXponential enrichment
SPE	Screen Printed Electrode
SPR	Surface Plasmon Resonance
TBE	Tris Borate EDTA

TCEP	Tris(2-carboxyethyl) phosphine
TMB	3,3’,5,5’-tetramethylbenzidine
UPEX	Universal Prolamin and glutelin EXtractant solution

Introduction

1.1 Celiac Disease

Celiac disease (CD) is an autoimmune pathology,[1] which affects about 1% of the global population.[2] It is an inflammatory process which causes damage to the upper small intestine and it is most prevalent in Europeans and those with European ancestry, but it is increasingly found also in areas such as North Africa, Middle East, and India.[3]

Consumption by celiac people of food containing gluten causes the damage of the mucosal villi and can lead to a flattened mucosa (see Figure 1.1),[4] resulting in malabsorption of nutrients and deficiency-related illnesses.[5]

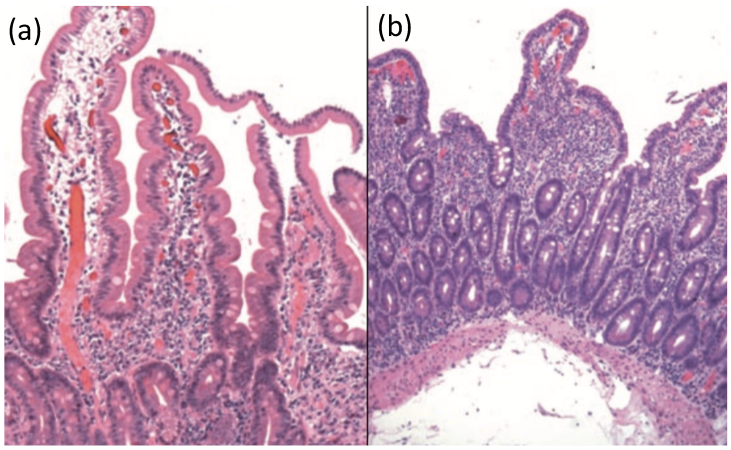

Figure 1.1 Normal duodenal mucosa (a) and duodenal mucosa in celiac disease (b).[4]

The term "gluten" refers to the entire protein component of wheat, while gliadin is the alcohol-soluble fraction of gluten containing toxic components. Gluten is a protein rich in glutamine and proline and it is poorly digested in the human upper gastrointestinal tract.[6] Undigested molecules of gliadin, such as a peptide from an α-gliadin fraction made up of 33 amino acids, called 33-mer,[7] are resistant to degradation by gastric, pancreatic, and intestinal brush-border membrane proteases in the human intestine. Thus, they remain in the intestinal lumen after gluten ingestion (see Figure 1.2) and constitute the main allergenic molecules for celiac people.[8]

The immune response causing the inflammatory reaction in the upper small intestine is mediated by both the innate and the adaptive immune systems. Typical symptoms of celiac disease are chronic diarrhea, vomiting, abdominal distension and others, such as anemia, deficiency of vitamins and minerals and loss of body mass, which are caused by malabsorption. Moreover, CD can also cause amenorrhoea, miscarriages or intrauterine growth retardation.[9]

Genetic factors are also involved in the development of the disease, in fact, there is a strong relationship between certain profiles of the HLA (human leukocyte antigen) system and the probability of suffering from CD.[10] HLAs are molecules that are found on the surface of white blood cells, which are involved in the recognition and signaling of the immune system. 90% of celiac patients possess the HLA-DQ2 molecule and 5% possess the HLA-DQ8.[11] However, not all genetically predisposed individuals will develop the disease.

Also environmental factors are responsible for the development of the illness.[12] In fact epidemiologic studies have pointed out that infants taking gluten before 4 months of age have an increased risk to develop the disease while the introduction of gluten in the diet after 7 months is associated with a marginal risk.[13]

Currently the only effective therapy available is a strict gluten-free diet, which means a permanent withdrawal of gluten from daily food.[14] Hence, people affected by celiac disease can eat naturally gluten-free products such as meat, fish, milk, fruits and vegetables or they can consume food specifically produced gluten-free, which are products like bread, pasta and beer.

These kinds of dietetic foods are regulated by the "Codex Alimentarius standard for foods for special dietary use for persons intolerant to gluten". This document sets a limit value of 20 mg/kg of gluten in products labeled "*gluten-free*".

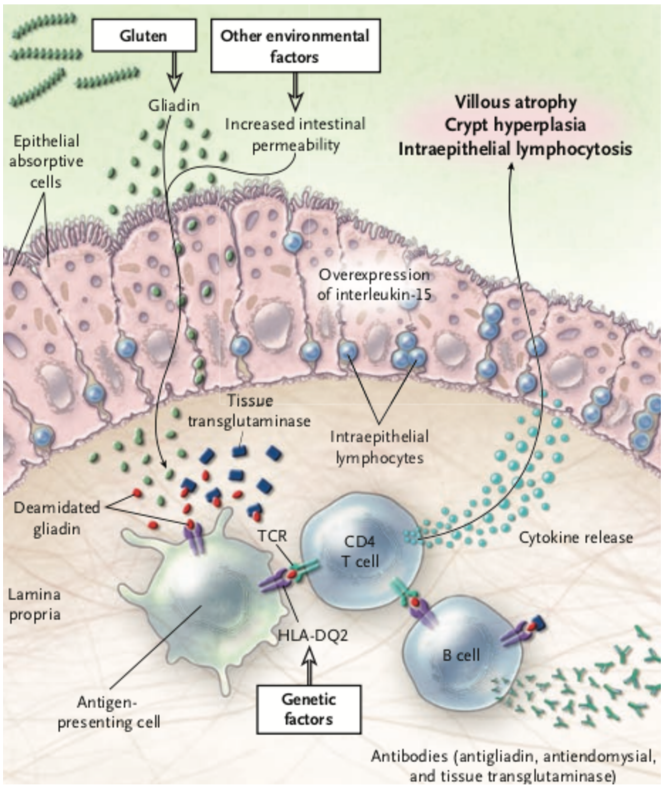

Figure 1.2 Interaction of gluten with environmental, immune, and genetic factors in celiac disease.[4]

1.2 Gluten detection as an analytical challenge

Considering celiac people condition, it is important to ensure that the gluten content in *"gluten-free"* products is below 20 ppm as set by the European Commission.[15]

To guarantee the safety of gluten-free food, the content of gluten has to be carefully determined. Quantification of gluten is usually performed with immunoassays and in recent years more than 30 kits have been commercialized (see Table 1.1).[16] Nevertheless, difficulties in accurate gluten quantification has not yet been overcome.[17,18]

For gluten quantification, the alcohol-soluble proteins, called prolamins (wheat gliadins, rye secalins, barley hordeins), are extracted with ethanol/water solutions 70% (v/v) or patented cocktail solutions and quantified by means of an immunochemical method, usually enzyme-linked immunosorbent assay (ELISA), exploiting one of the many antibodies against gluten proteins.[19] However, other techniques have also been employed such as mass spectrometry[20] and PCR-based methods.[21]

Undoubtedly one of the main problems in tackling gluten determination is to establish a precise target. In fact, since gluten is a set of proteins, it is not a specific analyte. Prolamines are used as reference (content is generally 50%); gluten is then calculated as the double of the quantity of prolamins.

Another relevant problem is the lack of a reference material. Currently, the most used standard is the PWG-Gliadin, which has been developed by a working group named the Prolamin Working Group with the aim of obtaining an internationally accepted reference material.[22] This group used 28 different European wheat crops to average their prolamin content, minimizing the variability. Although PWG-Gliadin is not a certified reference material, it is still the most commonly used material for the calibration and validation of analytical methods.

Moreover, extraction of gluten from food is a complicated goal since during thermal processing in the cooking and baking of food, several changes take place in these proteins.[23] Some of the changes that take place prevent the quantitative extraction of proteins, thus affecting their subsequent analysis.

Certainly, the problems regarding the full extraction of gluten proteins from food, the choice of the relevant epitopes and lastly the lack of a standardized reference material[24] make the determination of gluten still a challenge to be solved and an open topic in which research advances are advisable.

Manufacturer	ELISA kit	Principle	Antibody
Abnova	Gluten/Gliadin ELISA Kit	Sandwich	pAb
Astori Tecnica	Gluten ELISA Kit	Competitive	pAb
Biomedal Diagnostics	GlutenTox ELISA Sandwich	Sandwich	A1/G12 mAb
	GlutenTox ELISA competitive	Competitive	G12 mAb
	GlutenTox Sticks	Dipstick	G12 mAb
Biocontrol	Transia Plate Prolamins	Sandwich	R5 mAb
BioCheck (UK)	Gluten-Check ELISA kit	Sandwich	401.21 mAb
Diagnostic Automation	AccuDiagTM Gliadin/Gluten ELISA	Sandwich	pAb
ELISA Systems	ELISA Systems Gliadin assay	Sandwich	401.21 mAb
ELISA Technologies	Gluten Aller-Tek	Sandwich	401.21 mAb
	EZ gluten®	LFD	401.21 mAb
Elution Technologies	Gluten Rapid Kit	LFD	pAb
EuroProxima	Gluten-Tec® ELISA	Competitive	a20 mAb
Immunolab	Gliadin/Gluten	Sandwich	pAb
Imutest	Gluten-Check ELISA Kit	Sandwich	401.21 mAb
	Gluten-in-Food Test	Screening test	401.21 mAb
InCura	GlutenAlert ELISA	Competitive	pAb
Ingenasa	Ingezim Gluten®	Sandwich	R5 mAb
	Ingezim Gluten® SemiQ	Sandwich	R5 mAb
	Ingezim Gluten® Hidrolizado	Direct	R5 mAb
Morinaga Institute	Wheat Protein ELISA Kit	Sandwich	pAb
Neogen	Alert for Gliadin	Screening test	401.21 mAb
	Alert for Gliadin	Screening test	R5 mAb
	BioKits Gluten Assay Kit	Sandwich	401.21 mAb
	Veratox® for Gliadin	Sandwich	401.21 mAb
	Veratox® for Gliadin R5	Sandwich	R5 mAb
	Reveal 3-D for Gluten	LFD	401.21 mAb
R-Biopharm	Ridascreen® Gliadin	Sandwich	R5 mAb
	Ridascreen® Fast Gliadin	Sandwich	R5 mAb
	Ridascreen® Gliadin competitive	Competitive	R5 mAb
	Rida®Quick Gliadin	Dipstick	R5 mAb
Romer Labs	AgraQuant® ELISA Gluten G12	Sandwich	G12 mAb
	AgraQuant® ELISA Gluten	Sandwich	pAb
	AgraStrip® LFD Gluten G12	LFD	G12 mAb
	AgraStrip® LFD Gluten	LFD	pAb
Zeulab	Proteon Gluten Express	Dipstick	G12 mAb

LFD: lateral flow device; mAb: monoclonal antibody; pAb: polyclonal antibody; 401.21 mAb is also known as Skerritt mAb

Table 1.1 List of commercially available ELISA kits for gluten detection.[25]

1.3 Classification of wheat gluten proteins

Gluten contains hundreds of protein components which can be present as monomers or, linked by interchain disulphide bonds, as oligomers and polymers. It is the protein fraction remaining after wheat dough washing in order to remove starch, albumins and globulins.[26] Normally, gluten proteins are divided according to their solubility in alcohol-water solutions (e.g. 60% ethanol) in solubles gliadins and insoluble glutenins (see Table 1.2).[27] Gliadins and glutenins provide the rheological properties of dough. Gliadins contribute mainly to the viscosity and extensibility of the dough system. In contrast, glutenins are responsible for dough strength and elasticity.

Gliadins were first classified according to their electrophoretic mobility in a α, β, γ, ω-gliadins in order of decreasing mobility. α, β and γ-gliadins have overlapping MWs (28,000–35,000),[28] while glutenins form polymers with molecular weights from 80.000 Da to more than 10 million.

Another way of classifying wheat proteins is based on their amino acid sequence: sulfur-rich fraction, to which the α, β and γ-gliadins belong as well as aggregated gliadins, γ-secalins and β-hordeins; sulfur-poor fraction to which the ω-gliadins belong, the ω-secalins and the C-hordeins and the high molecular weight fraction.

Osborne-Goesaert Fraction	Solubility behavior	Composition	Biological role	Functional role
Albumin	Water and dilute buffers	Non-gluten proteins (mainly monomeric)	Metabolic and structural proteins	Protection from pathogens
Globulin	Dilute salt	Non-gluten proteins (mainly monomeric)	Metabolic and structural proteins	Providing food reserve to embryo
Gliadin	Aqueous alcohols	Gluten proteins (mainly monomeric gliadins and low molecular weight glutenin polymers)	Prolamins-type seed storage proteins	Dough viscosity/plasticity
Glutenin	Dilute acetic acid	Gluten proteins (mainly HMW glutenin polymers)	Prolamins-type seed storage proteins	Dough viscosity/plasticity

Table 1.2 Classification of proteins according to Osborne,[29] modified from Goesaert et al.[30]

An important aspect is certainly the identification of the various toxic peptides present in the different fractions which can be considered as targets for the analytical methods to be developed.
The N-terminus of wheat α-gliadin is known to be the most toxic to celiac patients. Sequences QQPFP, QQQFP, LQPFP, and QLPFP have been identified as the strongest target epitopes and are present not only in α/β-gliadins but also in the γ-type.[31] 33-mer peptide, corresponding to fragment QLQPFPQPQLPYPQPQLPYPQPQPF, is recognized as one of the most toxic peptide of gluten.[7]
Homologous sequences to these have been identified in the storage proteins of all cereals toxic for celiacs, while they are absent in cereals considered safe. These peptides are therefore usually used as targets to quantify the level of food toxicity.

1.4 Gluten extraction from food

One of the main problems of gluten detection is the extraction step. In fact, detection of gluten in products like bread and pasta and in products containing partially hydrolyzed gluten, such as beer, is particularly challenging.[25] The extraction of gluten proteins from food matrices should be as accurate as possible in order to avoid overestimation, or more seriously for celiac people, underestimation of prolamin content.
The most commonly used solvent in gluten quantification methods is water-alcohol solutions (60%), which is able to extract the prolamin fraction from non-processed food such as flour. However, this method is not able to completely extract gluten from processed food, because prolamins and glutelins aggregate by interchain disulphide bonds formed during heat processing.[32]
In order to overcome this problem, reducing agents, such as 2-mercaptoethanol, and disaggregating agents, such as guanidine or sodium dodecyl sulphate, have been used in combination with alcohol solutions.[25]
The need for improved sample extraction methods led to the development of the patented cocktail solution of Prof. Dr. Enrique Mendez employed in many ELISA kits for the extraction at 50°C.[33] This cocktail solution contains 2-mercaptoethanol and guanidine in a phosphate buffer.[34] Another extraction solution is the UPEX (universal prolamin and glutelin extractant solution) containing the reducing agent Tris(2-carboxyethyl) phosphine (TCEP) and the surfactant N-lauroylsarcosine.[35]

It has to be also considered the nature of the food matrix, because additional steps may be necessary, such as defatting for products containing for instance chocolate or the addition of fish gelatin for products with high concentrations of polyphenols.

However, both reducing agents and denaturants used in the extraction cocktails can cause interference in the subsequent protein recognition, affecting the results of the quantification.[36] Of course, it is of great importance to verify the compatibility of the extraction solvent with the analytical procedure chosen to quantify gluten.

Problems concerning extraction methods still have not been fully overcome. Thus, there is the need to find a universal effective solution compatible with bioassays and in particular with the recognition element employed in the method.

1.5 Gluten quantification: ELISA assays

To ensure the safety of products for celiac disease patients, foods labeled gluten-free must not exceed the level of 20 mg gluten per kg of product. This sets the standard for analytical methods for gluten detection. Until now, enzyme-linked immunosorbent assays (ELISA), which are analytical bioanalytical assays based on the use of antibodies, are recommended by legislation.

ELISAs are most commonly used for gluten analysis not only for their selectivity and sensitivity but also for the lack of other reference methods. Alternatives to ELISA are currently developing like proteomics, mass spectrometry, genomics and novel approaches such as aptamers-based biosensors and nano or microarrays with electrochemical transduction.

In general, immunoassays use a specific antibody to detect the antigen, which is the specie to be determined. Usually, ELISAs exploit detection antibodies that are covalently linked to an enzyme, such as horseradish peroxidase, to increase detectability. Two main set up of ELISA are available for gluten analysis: sandwich and competitive.

In a sandwich ELISA the plate is coated with the capture antibody. The sample containing the antigen is then added and bound to capture antibodies. Afterward, the detecting antibody is added and binds to a second binding site of the antigen (see Figure 1.3). At this point, the antigen is "*sandwiched*" between two antibodies. The addition of the enzymatic substrate that is transformed

into a coloured product, whose absorbance can be measured in a plate reader, allows the analyte to be quantified. The absorbance is directly proportional to the antigen concentration.

This kind of assay is suitable for large antigens since there is the need to have two separate binding-site for the interaction with two antibodies. Hence, for products such as beer in which gluten is partially hydrolyzed, this kind of assay is inappropriate.

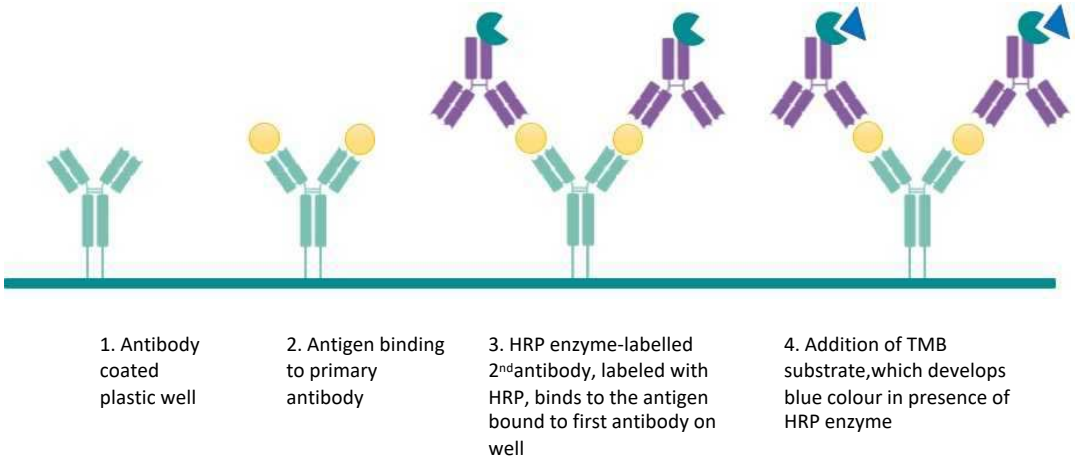

Figure 1.3 Schematic representation of a sandwich ELISA.

In a competitive ELISA assay, a known antigen amount is immobilized on the plate surface. The sample, containing the antigen, and a constant amount of antibody labelled with an enzyme are added simultaneously to the well. During the incubation time, the immobilized and the free antigens compete to bind the antibody. More antigens are present in the sample, fewer antibodies will be bound to the immobilized antigens (see Figure 1.4). After washing, the addition of the enzymatic substrate generates a coloured product. In this case, the absorbance is inversely proportional to the antigen concentration. This assay may be used for both intact proteins as well as gluten hydolized peptides.

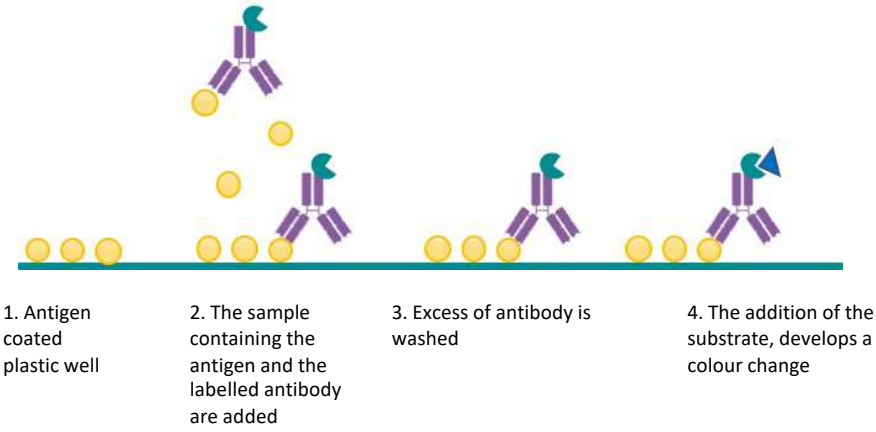

Figure 1.4 Schematic representation of a competitive ELISA.

Most of the kits for gluten detection are based on the use of antibodies such as the Skerritt (known also as 401.21),[37] R5,[33] G12,[38] α20[39] and various polyclonal antibodies.

The Skerritt monoclonal antibody was raised against ω-gliadins and it recognizes the epitopes PQPQPFPQE and PQQPPFPEE.[37] The R5 monoclonal antibody was raised against ω-secalins and it interacts with the epitopes QQPFP and the related sequences QQQFP, LQPFP and QLPFP.[40] The G12 antibody was produced against the immunotoxic 33-mer peptide and reacts with the epitope QPQLPY.[38] The monoclonal antibody α20, was generated against the CD-immunogenic peptide PFRPQQPYPQP from α -gliadins.

The sandwich R5 ELISA together with cocktail extraction was adopted as AACCI (American Association of Cereal Chemists International) Approved Method.[38] It is also endorsed by the AOACI (Association of Official Analytical Chemists International) as official method and by the Codex as a type 1 method for the analysis of intact gluten in corn-based matrices. A competitive R5 ELISA was developed for the determination of partially hydrolyzed gluten and accepted as AACCI Approved Method. The sandwich G12 ELISA was adopted as AACCI Approved Method and it was added as a type 1 method for the analysis of intact gluten in rice-based matrices by the Codex in 2015.

1.6 Aim and objectives

As discussed earlier, regardless of all the advances that have occurred so far, the development of gluten quantification methods still encounters significant difficulties derived from the identity of the allergen, the lack of an effective and universal extraction method and the availability of few receptors of sufficient affinity and selectivity to obtain a sound analytical method.

The use of deep eutectic solvents (DESs) as extractants is an expanding field as well as the use of aptamers as recognition elements for the development of analytical methods in food safety. Combining these two aspects could bring advances in the correct gluten content evaluation in food and represents at the same time an innovative approach to be applied to other analytes.

Therefore, the aim of this thesis was the development of a new detection method for gluten by coupling DESs with aptamers. With this main aim, the objectives of the thesis were:

- The investigation of deep eutectic solvents as innovative extraction media for gluten proteins.
- The selection by SELEX of aptamers targeting gluten in the deep eutectic solvent with the best extraction capability.
- The characterization and affinity determination of selected aptamers.
- The development of an effective quantification method for gluten based on aptamers in deep eutectic solvent.

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Extraction of gluten proteins by deep eutectic solvents (DESs)

2.1 Deep eutectic solvents (DESs)

Recently, a novel class of non-acqueous ionic liquid solvents, called deep eutectic solvents (DESs), has emerged. DESs are prepared by mixing at least two safe components able to form an eutectic mixture with a melting point lower than that of starting components (see Figure 2.1).[1] DESs share similar properties with ionic liquids (ILs), a class of organic salts with a low melting point, usually obtained by combining an organic cation (commonly imidazolium-based cations) with a large variety of anions (i.e. Cl^-, BF_4^-, PF_6^-, NTf_2).[2]

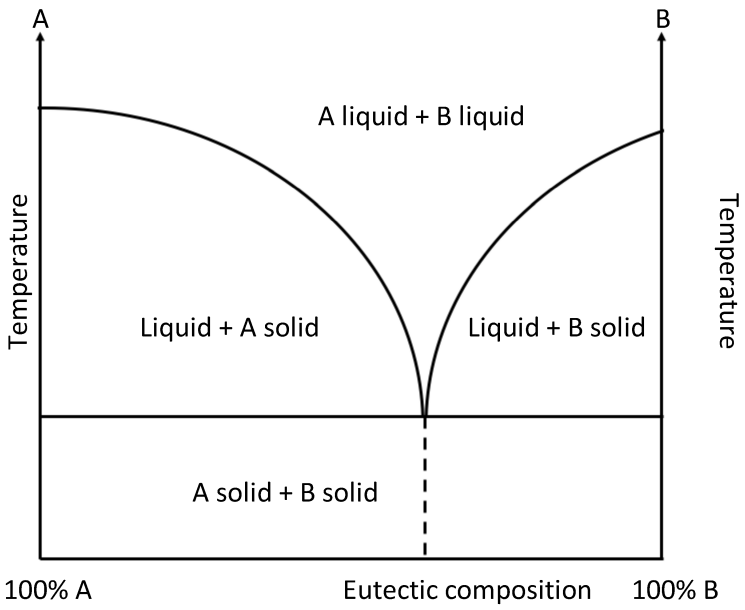


Figure 2.1 Eutectic phase diagram.

ILs received growing attention due to their chemical and physical properties that made them attractive solvents compared to conventional organic solvents.[3] In fact, they display negligible vapor pressure, good thermal stability and electrical conductivity, tuneable miscibility with water and organic solvents, combined with the ability to dissolve a large number of organic and inorganic compounds. However, their definition as "green" species is largely contested in the current literature.[4] Indeed, ionic liquids can't be completely defined as green on the basis of the twelve green chemistry principles.[5] Therefore, in order to overcome the high cost of their production and their toxicity, DESs have arisen as a cheaper and greener alternative.[6]

DESs are generally obtained by mixing a hydrogen bond acceptor (HBA), such as quaternary ammonium salts, with a hydrogen bond donor (HBD), such as amines, carboxylic acids, alcohols, or carbohydrates (see Figure 2.2).[7] They were firstly reported in 2004 by Abbott and they have been

applied in many different fields such as organic synthesis, electrochemistry and bio-catalysis.[8] Moreover, they display some interesting advantages, such as improved biodegradability and lower toxicity.[9]

A further interesting aspect is that the occurrence of DESs in living organisms could explain the presence of poorly water or lipids-soluble molecules in cells.[10] As a matter of facts, natural products represent ideal sources for DESs due to their chemical diversity and biodegradability.

Different studies have led to the discovery of a novel class called Natural Deep Eutectic Solvents (NADES).[11] This new class of solvents may explain mechanisms and phenomena occurring in living cells that are otherwise difficult to understand, such as the biosynthesis of non-water-soluble small molecules and macromolecules as well as the presence in cells and organisms of poorly water or lipids-soluble molecules. The presence in large amounts in all microbial, mammalian, and plant cells of some compounds such as sugars, some amino acids, choline, and some organic acids such as malic acid, citric acid, lactic acid, could lead to the formation of NADES as a third type of solvent besides water and lipids.

Both DESs and NADES provide a network of hydrogen bonds that make possible the solubilization of a wide range of molecules.[12] They are potentially able to act as effective solvents for the extraction of a wide range of non-polar and polar compounds.[13] Thus, they can be an alternative to several conventional and toxic organic solvents.[14]

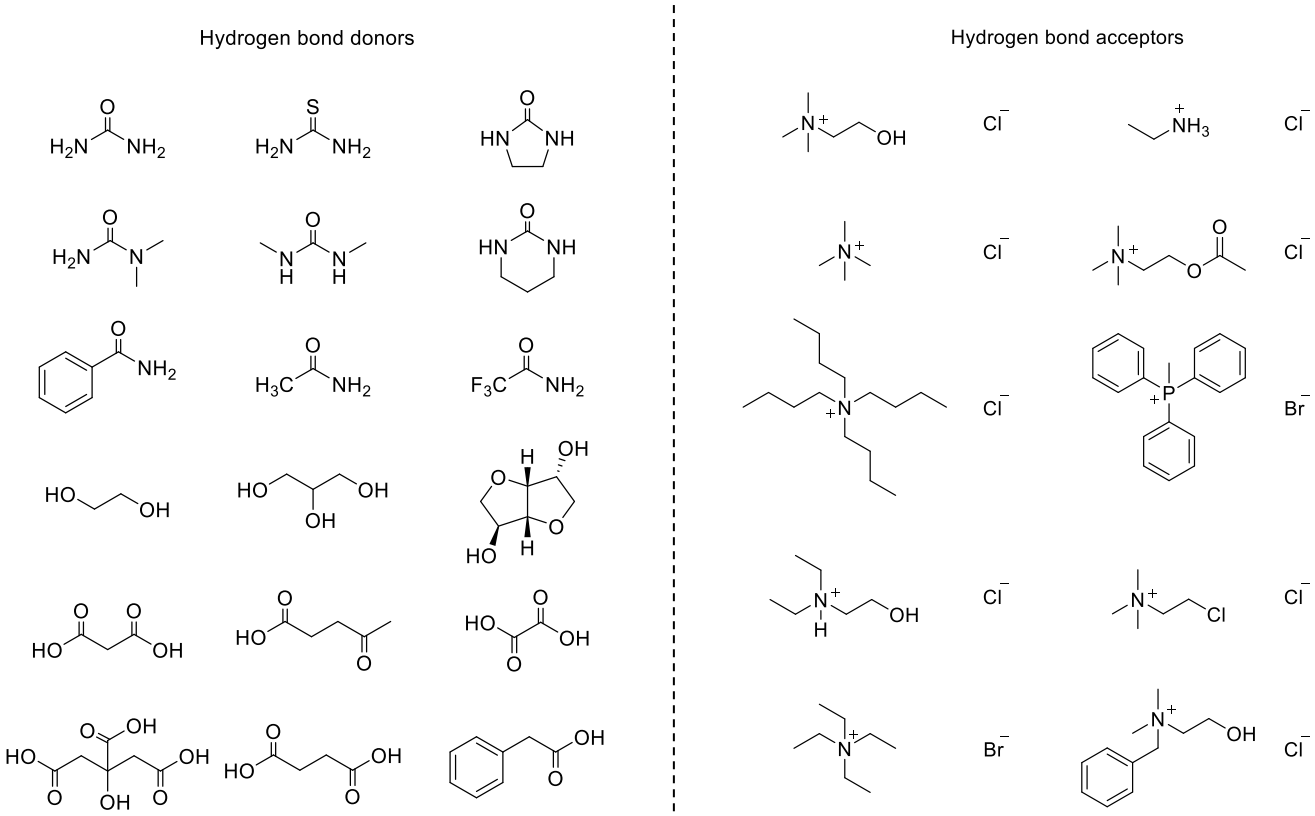


Figure 2.2 Typical hydrogen bond donors and hydrogen bond acceptors employed in DESs.

2.1.1 Physicochemical properties

DESs are easily produced by mixing two or more compounds and heating them to about 80 °C.[15] DESs physicochemical properties, such as melting point, density, conductivity, and viscosity, vary depending upon their structures. In general, the high viscosity of DESs is due to the presence of an extensive hydrogen-bonding network between the compounds that restricts the mobility of free species inside the solvent. Other interactions, such as van der Waals and electrostatic interactions, may also contribute to the high viscosity of DESs. The addition of water and the modification of DES constituents have always been used to overcome this disadvantage.[16] However, it must be noted that the addition of water excess may lead to the weakening of hydrogen bonding interactions between DES constituents.

The freezing point of DESs depends on their components (type of HBD and HBA) and their molar ratio. They exhibit in general higher densities than water, with values ranging from 1.041 g cm^{-3} to 1.63 g cm^{-3}.[9]

Among different quaternary ammonium salts employed in eutectic mixtures, choline chloride is the most employed owing to its low cost and biodegradability. Moreover, it can be combined with

different hydrogen bond donors (HBD) to provide DESs with different physicochemical properties such as freezing point, viscosity, conductivity, and pH.[17] Table 2.1 shows some physical parameters of choline-chloride based DESs.

DESs	**salt/HBD molar ratio**	**viscosity[a] Pa·s**	**conductivity[a] $\mu S \cdot cm^{-1}$**	**density[a] g/cm^3**
ChCl/acetamide	1:2	0.127	2710	1.0852
ChCl/glycerol	1:2	0.177	1647	1.1854
ChCl/1,4-butanediol	1:4	0.047	2430	1.0410
ChCl/triethylene glycol	1:4	0.044	1858	1.1202
ChCl/xylitol	1:1	3.867	172.6	1.2445
ChCl/D-sorbitol	1:1	13.736	63.3	1.2794
ChCl/oxalic acid	1:1	0.089	2350	1.2371
ChCl/levulinic acid	1:2	0.119	1422	1.1320
ChCl/malonic acid	1:1	0.616	732	1.2112
ChCl/malic acid	1:1	11.475	41.4	1.2796
ChCl/citric acid	1:1	45.008	18.4	1.3313
ChCl/tartaric acid	2:1	66.441	14.3	1.2735
ChCl/xylose/water	1:1:1	0.887	1092	1.2505
ChCl/sucrose/water	5:2:5	3.939	147.2	1.2737
ChCl/fructose/water	5:2:5	0.598	1399	1.2095
ChCl/glucose/water	5:2:5	0.584	2820	1.2094

[a] Determined at 30 °C.

Table 2.1 Physical Parameters of DESs

To date, DESs have found many applications in analytical chemistry, such as extraction of analytes from complex liquid and solid matrices, modification media for nanoparticles, elution in dispersive solid phase extractions and as mobile phase modifier in chromatography. All these applications show the great ability of DESs to act as solubilizing media and their potential use in many chemistry fields.

The adjustable physicochemical properties of DESs, such as their viscosities and polarities, represent an advantage that can be exploited in extraction processes. In fact, DESs polarity can be adjusted to either polar or non-polar, thus making them efficient solvents for the extraction of different classes of compounds. Lately, they have been used to extract bioactive compounds,[18] such as flavonoids, phenolic acids, polyphenols, saponins, and anthraquinones from various types of natural sources.[19–22] In addition, DESs are considered for their good solubility toward many other compounds, including drugs, metal oxides, and carbon dioxide.[23,24] Eutectic solvents have also been reported as extraction or dissolution media for some biopolymers such as lignin, cellulose and starch.[25] Their excellent dissolution capability is due to the fact that they are able to donate and accept both protons and electrons, thus facilitating the formation of hydrogen bonds between molecules. Hence, the possibility of employing DESs with different polarities makes them able to dissolve several non-water-soluble metabolites or macromolecules. Therefore, they could be suitable for protein extraction from complex matrices, such as food.

2.2 Aim of this work: gluten extraction with DESs

The aim of this part of the thesis was to develop an alternative to well-known extraction methods for gluten, which are usually non-compatible with bioassays requiring the use of large sample dilutions prior to analysis. So, in order to overcome this problem, we decided to develop a new gluten extraction method exploiting the properties of eutectic liquids.

2.3 Experimental

2.3.1 Reagents and Apparatus

We tested the ability of two choline chloride DESs, ethaline and reline, to extract gluten from food, as well as their compatibility with a commercially available enzyme immunoassay. Ethaline and reline were chosen for these tests because they display a moderate viscosity and do not present handling difficulties at room temperature. They were supplied by Scionix Ltd. (London, UK) and were employed as received, without further purification. Table 2.2 reports their formula, together with their viscosity at 25 and 55 °C from literature data[26] and water content determined by us with a Karl-Fischer titration.

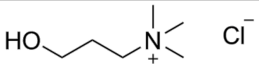
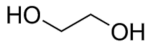
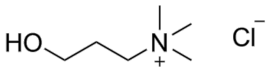
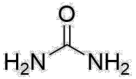
ChCl-DES	Salt-HBA	HBD	Molar ratio	Viscosity (25°C) mPa s	Viscosity (55°C) mPa s	Water content
Ethaline	 choline chloride	 ethylene glycol	1:2	37	24	2.7 %
Reline	 choline chloride	 urea	1:2	750	95	3.1 %

Table 2.2 Formula and component molar ratios of ChCl-DESs adopted.

Both ethaline and reline display at 25 °C a viscosity which is quite higher than that of common gliadin extractants (from 30 to 1000 times higher than water), but it becomes considerably lower at 55 °C which is the temperature usually recommended for gluten quantification, at which no protein denaturation occurs.[27] This temperature-induced viscosity decrease of DESs is expected to improve their extraction efficiency since analyte diffusion becomes quicker.

Ethanol used to prepare 60% (v/v) ethanol-water extraction solutions was purchased from Sigma-Aldrich (Milan, Italy). Reference gliadin (90.8% w/w purity) from the Prolamin Working Group (PWG gliadin) was supplied by the Group on Prolamin Analysis and Toxicity (Germany) and used as the standard. A 1000 mg/L PWG gliadin standard solution was prepared in 60% (v/v) ethanol-water.

Gluten in food samples was quantified by the ELISA immunoassay test performed by using the Gluten-Check™ kit supplied by Bio-Check (UK). Water used as solvent was ultrapure water, purified by an Elgastat UHQ PS system (ELGA LabWater, Siershahn, Germany). Both unheated (flour) and heated (biscuits and crackers) gluten-free foods were purchased from local supermarkets.

An ultrasonic bath from Bransonic Co. (USA) was used to prepare high-concentration PWG gliadin standard solutions. ELISA spectrophotometric measurements were carried out with a PerkinElmer VICTOR3 1420 Multilabel Counter absorbance microplate spectrophotometer (Waltham, MA, USA), while an Agilent Cary 50 UV-Vis spectrophotometer (Santa Clara, CA, USA) was used to record UV-Vis spectra. An analytical balance CP Sartorius (Sartorius, Goettingen, Germany) was used to weight samples. In agreement with the procedure recommended by the Gluten-Check™ ELISA kit, all solid samples were grinded, when necessary, by a Sterilmixer homogenizer (International PBI, Milan, Italy) and shaken using a vortex (International PBI, Milan, Italy). A water bath (Werke GmbH & Co. KG, Staufen, Germany) and a Thermo Scientific Heraeus Labofuge 200 Centrifuge (Waltham, MA, USA) were used for sample extraction and centrifugation, respectively.

2.3.2 Sample Preparation and Extraction Procedure

Gluten was extracted with ethaline and reline and for the sake of comparison, by usual 60% (v/v) ethanol-water solutions.

About 50 g of each food sample was milled in a grinder to prepare a fine powder. Afterward, 0.35 g of the obtained powdered sample was extracted in vials with 3.5 mL of 60% (v/v) ethanol-water solution or pure DES. These vials were shaken by a vortex for 2 min, and then they were left in a water bath at 55 °C for 45 min. After this time, they were shaken again for 2 min and centrifuged for 10 min at 5000 rpm. Similar aliquots of powdered food were also spiked with known amounts of PWG-Gliadin (leading to spiked contents ranging from 5 to 36 mg/kg) and subjected to the same treatment described above. After extraction, 100 µL of supernatant was diluted (10:1 or 20:1) with the dilution solution provided by the ELISA kit. Sample preparation was carried out in a laboratory separated from that where analyses were performed, to avoid contamination. All extractions were carried out in triplicate.

2.3.3 ELISA quantification

Gluten content in food samples was determined by the Gluten-Check™ ELISA. This test is a two-step method which employs the 401/21 antibody developed by Skerritt and Hill,[28] approved by the Association for Official Analytical Chemists (AOAC) for the quantification of gluten in food.

After dilution with the kit solvent, extracted samples were incubated with the 401/21 monoclonal antibody immobilized onto the bottom of the plate's wells. After incubation for 20 min, wells were emptied and washed three times with the washing solution supplied by the ELISA kit (phosphate-buffered ethanol-water). Then, the solution containing the antibody labeled with the enzyme horseradish peroxidase (HRP) was added. In this way the analyte formed an adduct between the antibody attached to the well and antibody labeled with the enzyme. After other 20 min, the excess of non-immobilized labeled antibody was removed by using the kit washing solution (four repeated washings). Then, the solution containing 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and kept in the dark, so that the enzyme oxidizes the TMB, giving the corresponding blue-colored oxidized form (see Figure 2.3).

The enzymatic reaction was hence stopped by acidification with 0.5 M H_2SO_4 which caused simultaneously inhibition of the enzyme and conversion of the oxidized form of TMB to the corresponding yellow-colored protonated form.

A calibration curve was constructed by plotting the absorbance at 450 nm against the gluten concentration of five standard solutions, in the range 5–110 mg/L, provided by the ELISA kit.

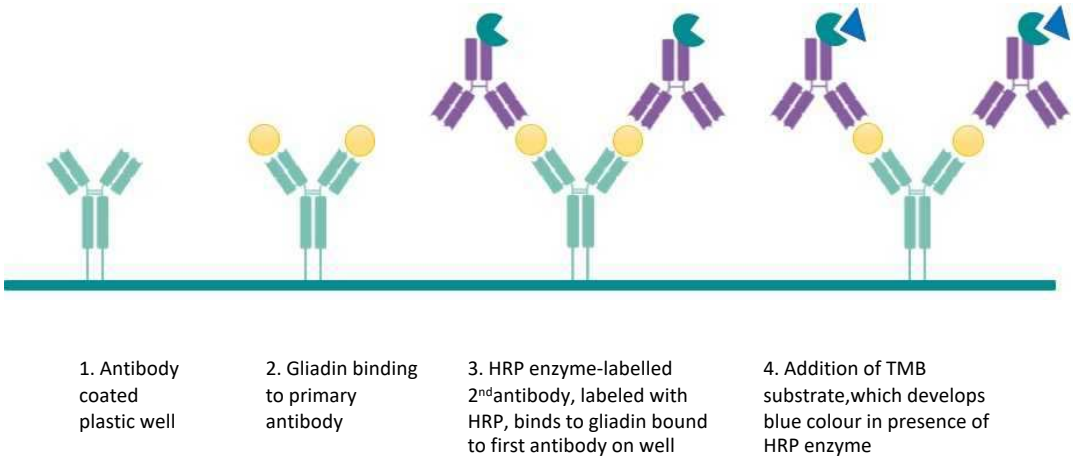


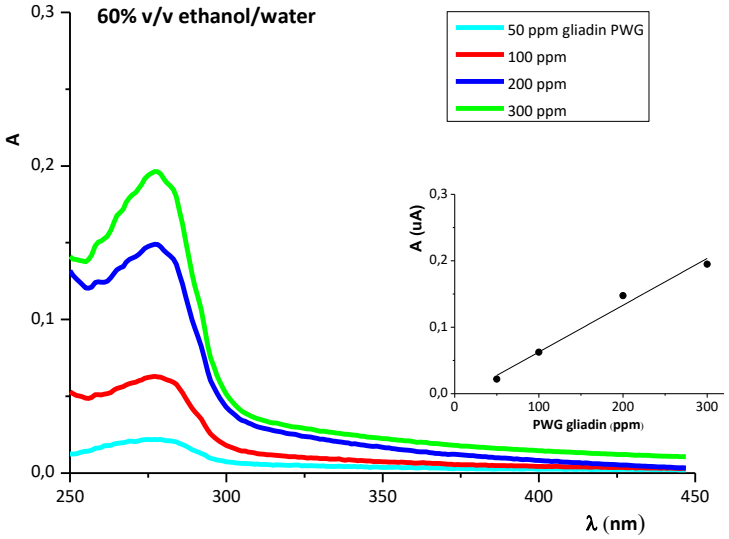
Figure 2.3 Diagram of ELISA method.

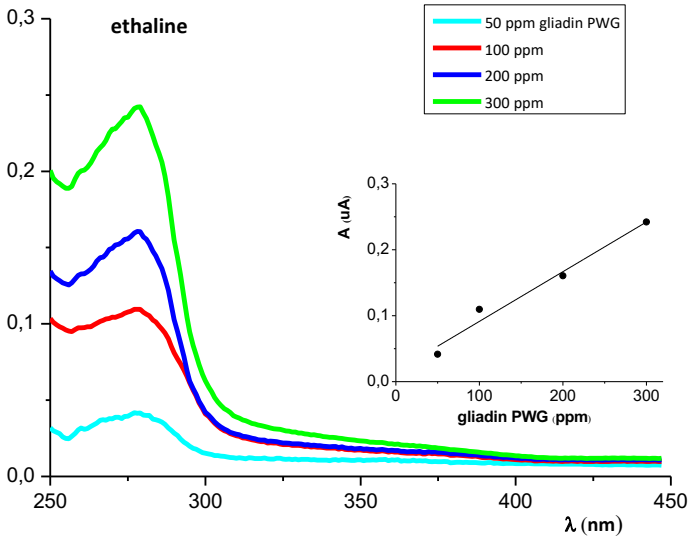
2.4 Results and Discussion

2.4.1 Solubility tests of PWG Gliadin in ChCl-DESs

The ability of ethaline and reline to dissolve PWG-Gliadin was verified performing some preliminary tests. With this purpose, a series of solutions containing PWG-Gliadin, in each DES at 25 °C, with concentrations ranging from 50 to 300 mg/L were prepared. Concomitantly, for the sake of comparison, PWG-Gliadin solutions with the same concentrations were also prepared in 60% (v/v) ethanol-water. All standard solutions turned out to be clear, without precipitates, they were subjected to absorbance measurements in the UV-Vis range 250–450 nm, where proteins display an absorption band with a maximum at about 280 nm.[29] UV-Vis spectra, were recorded with an Agilent Cary 50 UV-Vis spectrophotometer (Santa Clara, CA, USA). These spectra are summarized in Figure 2.4. They showed in all cases a linear increase with the PWG-Gliadin concentration. In particular, spectra recorded in DES media did not show appreciable differences from those recorded in 60% (v/v) ethanol-water and also the corresponding calibration plots (insets in Figure 2.4) turned out to be similar enough. In fact, regression equations found in 60% (v/v) ethanol-water, ethaline, and reline, respectively, were the following: $A = 7.042 \times 10^{-4} x C_{PWG}$ (mg/L) − 0.008; $A = 7.535 \times 10^{-4} x\ C_{PWG}$ (mg/L) + 0.016; and $A = 8.017 \times 10^{-4} x\ C_{PWG}$ (mg/L) − 0.009.

When these tests were repeated in DESs at 55 °C, very similar UV-Vis spectra were once again recorded. In addition, when absorbance measurements in DES media were repeated every hour, the relevant spectra turned out to be repeatable for 1 day at least. These findings pointed out that DESs adopted by us are effective solvents for PWG gliadin and that they do not cause analyte degradation.


60% v/v ethanol/water
50 ppm gliadin PWG
100 ppm
200 ppm
300 ppm
A
λ (nm)
A (uA)
PWG gliadin (ppm)


ethaline
50 ppm gliadin PWG
100 ppm
200 ppm
300 ppm
λ (nm)
A (uA)
gliadin PWG (ppm)

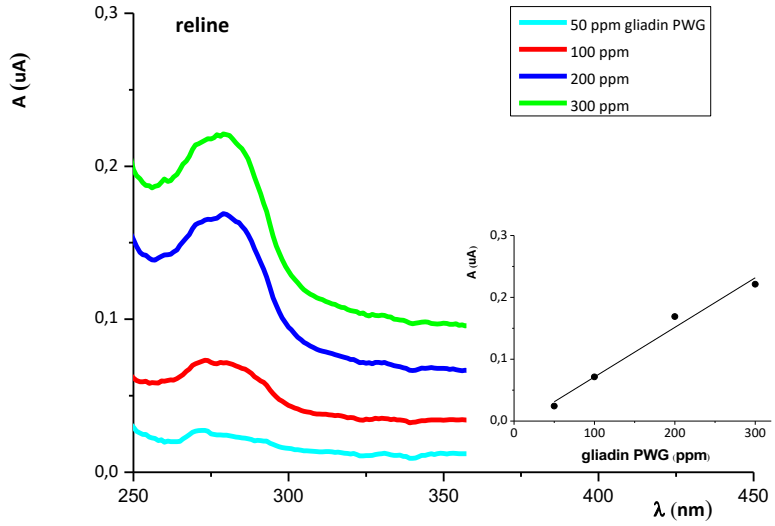


Figure 2.4 Absorption spectra, recorded at 25 °C, for PWG gliadin at different concentrations (from 50 to 300 mg/L) in 60% (v/v) ethanol- water, ethaline, and reline. Insets: PWG gliadin absorbance at 278 nm versus its concentration.

2.4.2 Endurance of the ELISA (Gluten-Check™) Assay for ChCl-DES Media

In principle, the performance of the Gluten-Check™ ELISA immunoassay employed by us for gluten determination could be interfered by the presence of DESs used in the extraction step. In fact, these media could change to some extent the gliadin environment, thus causing its conformational alteration and making the ELISA immunoassay no longer suitable for its determination. Consequently, some preliminary tests were performed to verify whether ethaline and reline were able to affect the detection procedure.

With this purpose, standard solutions of PWG gliadin in both ethaline and reline were prepared by adding controlled amounts of the analyte to the pure solvents, so as to achieve concentrations ranging from 0 to 50 mg/L.

100 µL of these solutions were then subjected to the detection procedure. The results of seven replicate measurements performed on each sample turned out to be satisfactory. In fact, PWG-Gliadin concentrations found turned out to be coherent with the expected contents, with a RSD of ±9%. This result confirmed that the replacement of ethanol-water by ethaline or reline as extraction media is fully compatible with gluten detection with this ELISA kit.

2.4.3 Determination of gluten in food samples

The ability of ethaline and reline to extract gluten from food was tested on real food samples. Both heat-untreated (flour) and heat-treated (crackers and biscuits) gluten-free food samples were assayed, in view of the fact that significant differences in gluten contents were found on passing from flour to the corresponding bread.[30] For the sake of comparison, all real samples were also subjected to extraction with 60% (v/v) ethanol-water. After extraction, these samples in both DESs and ethanol-water were subjected to analysis for their natural gluten content.

Moreover, four spiked samples were prepared by adding controlled amounts of PWG-Gliadin standard solutions to 0.35 g of both heat-treated and heat-untreated gluten-free food samples. Three replicates of each sample and of each spike level were tested. Also, these spiked samples were extracted with ethaline, reline and with ethanol-water, following the procedure previously described. After centrifugation, 100 µL aliquots of supernatants were analyzed by the ELISA kit.

The results obtained are summarized in Table 3, where recoveries achieved with the different solvents are compared. Even though gliadin found in spiked samples did not come from gluten, since equivalent amounts of glutelins were of course absent, all data reported in this Table are expressed as milligrams per kilogram of the corresponding gluten (double of gliadin mass), as inferred from the calibration curve provided by the ELISA kit.

Data in Table 2.3 show that when food samples characterized by small gluten concentrations were analyzed, such as unspiked gluten-free samples, all extraction solvents employed led to comparable gluten contents (a little more than 2 mg/kg).

In contrast, when spiked samples displaying higher gliadin concentrations (added amount plus natural content) were tested, the usual 60% (v/v) ethanol-water extraction solvent turned out to provide a quite modest performance. Better results were found when the extraction was conducted with the DES reline, but the best performance in terms of recovery was provided by ethaline. This ability of ethaline to act as a more effective extraction medium with respect to reline can be explained considering the lower viscosity displayed by this DES, which is expected to increase the analyte mass transfer rate from the sample to the extraction medium.

In particular, it is important to underline that gluten recoveries achieved by ethaline turned out to be totally satisfactory for samples spiked with gliadin amounts nearly 10 mg/kg (corresponding to about 20 mg/kg of gluten), which is the gluten limit between gluten-free and gluten-containing food samples.

The better performance offered by ethaline with respect to the 60% v/v ethanol-water solvent is consistent with the gliadin solubility in these different media. In fact, solubility tests performed by us for gliadin in ethaline at room temperature showed that PWG solubility was largely higher than 5000 mg/L, while a solubility of ca. 1600 mg/L was reported for gliadin in ethanol at room temperature. No literature report is so far available about gliadin solubility in 60% v/v ethanol-water at higher temperatures, but it is expected that a temperature increase causes a solubility increase in both ethanol-water and ethaline. Consequently, ethaline should remain conceivably a more effective solvent for PWG-Gliadin even at 55 °C.

Extraction solvent	**Gliadin spike mg Kg^{-1}**	**Gluten found (recovery% ± RSD%) mg Kg^{-1}**		
		Flour	**Crakers**	**Biscuits**
Ethaline	-	2.3 ± 0.1	2.4 ±0.1	2.1±0.2
	4.5	11 (93 ± 11)	12.9 (113 ± 12)	8.9 (80 ± 13)
	9.0	19 (94 ± 7)	19 (93 ± 7)	15.8 (78 ± 3)
	18	42 (109 ± 8)	-	-
	36	76 (102±12)	-	-
Reline	-	2±0.2	2.55±0.1	2.05±0.2
	9	14 (67 ±15)	17 (84 ± 6)	26.5 (132 ±20)
	18	28 (74 ± 8)	-	-
Solvent supplied by the Extraction kit	-	2.3± 0.1	2.05± 0.1	1.95± 0.1
	9	16 (76± 10)	15 (72 ± 7)	17 (84 ± 9)
	18	46 (121 ± 13)	-	-
(60% v/v) EtOH	-	2.3± 0.1	1.9± 0.2	1.9± 0.1
	9	5 (25 ± 20)	12 (60 ±15)	7 (36 ± 18)
	18	22 (58 ± 27)	-	-

Table 2.3 Recovery % ± relative standard deviation (RSD) for gluten-free and PWG gliadin spiked food samples.

2.5 Conclusions

The results achieved point out that DESs such as ethaline and reline are very promising as gluten extraction solvents from both unprocessed and processed food, because they provide reliable results. The use of these DESs is fully compatible with the subsequent gluten detection with a usual sandwich immuno-assay exploiting the 401/21 antibody, without any change of the procedure recommended by the ELISA kit adopted.

Both DESs provided a performance comparable enough with that offered by the usual 60% (v/v) ethanol-water solvent for samples containing small gluten contents, while they proved to be better extraction solvents when food samples characterized by higher gluten concentrations were analyzed. Ethaline acted as a more effective extraction medium with respect to reline and this can be explained on considering the higher viscosity of reline which causes the analyte mass transfer from the sample to the extraction solvent to be slowed down.

In particular, recoveries provided by ethaline from samples containing about 20 mg/kg of gluten (range where it is crucial to distinguish between gluten-free and gluten containing samples) proved to be more accurate than those provided by other extraction solvents so far adopted.

Finally, comparing our findings with those gained carrying out the gluten extraction from food by using sugar-based DESs,[31] recoveries found by us using ethaline and reline were almost comparable with those achieved in spiked real samples extracted by sugar-based DESs. However, extractions conducted with sugar-based DESs required their preliminary dilution with water in order to reduce their quite high viscosity. On the contrary, the low enough viscosity displayed at 55 °C by DESs, in particular by ethaline, allowed their direct use as solvent for gluten extraction.

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Aptamer selection in a Deep Eutectic Solvent (Ethaline)

3.1 Aptamers

Aptamers (from Latin *aptus* that means fit, and Greek *meros,* meaning part) are short single stranded sequences of oligonucleotides selected in vitro to bind a specific target.[1] For a long time, nucleic acids have been ascribed only the function of containing the genetic information required for the biosynthesis of RNA and proteins. However, in the 90s it was discovered that short RNA molecules could fold in three dimensional structures able to bind specific targets following the complementarity rules of nucleic bases, as shown in Figure 3.1.

So, an in vitro Darwinian procedure, called SELEX (Systematic Evolution of Ligands by EXponential enrichment), was developed in order to obtain sequences for specific targets and the word *aptamer* was coined, referred to ssDNA synthetic molecules.[2]

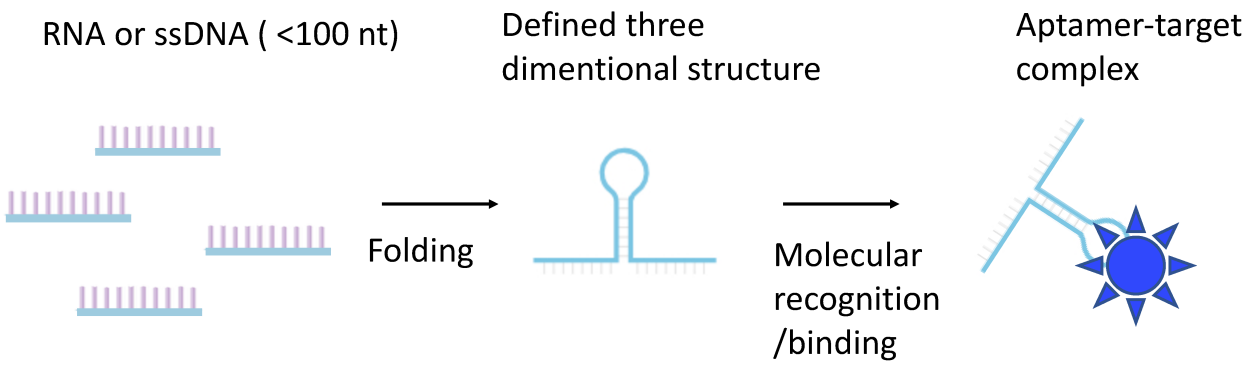


Figure 3.1 Schematic representation of aptamer–target interaction.

Thanks to their characteristics, aptamers are playing an important role in biotechnology. The fact that they can be selected with high affinity and specificity for a wide range of targets, such as ions, small molecules, proteins, viruses, bacteria and cells has made them a powerful tool in many research fields.[3]

They have been applied to many analytical techniques such as western blotting, flow cytometry, capillary electrophoresis, mass spectrometry and column chromatography.[4] Moreover, they are compatible with many different detection approaches, such as electrochemical, fluorescence, colorimetric, chemiluminescence, field effect transistors and surface plasmon resonance (SPR).[5]

They were also used in medical and radio-diagnostic,[6] as well as for therapeutic applications.[7]

In 2004, Macugen was the first aptamer approved by Food and Drug Administration (FDA) for the treatment of macular degeneration of eye-retinas.[8][9] However, their application still remains

underdeveloped, since no Food and Drug Administration approved clinical diagnostic test using aptamers is available on the market. There is not a clear reason to justify this. Even though aptamer selection, through SELEX, is time consuming and expensive, they are easy to prepare and manipulate. In fact, once the suitable sequence has been identified, they can be simply purchased from an online oligonucleotide vendor for a reasonable price and received in few days by express mail. Moreover, it worth noting that the SELEX process can be optimized, cost reduced, and it is also possible to choose among different SELEX approaches, such as magnetic bead-based, 2D Electrophoresis and Cell-SELEX. Furthermore, these approaches can be also performed against unpurified and difficult targets, obtaining a recognition system that otherwise would be almost impossible to develop.[10]

Furthermore, there is the evidence of a growing interest for this topic. In the last ten years, the scientific literature production concerning aptamers has remarkably grown, as shown in Figure 3.2, where the trend with time of publications concerning aptamers is reported.

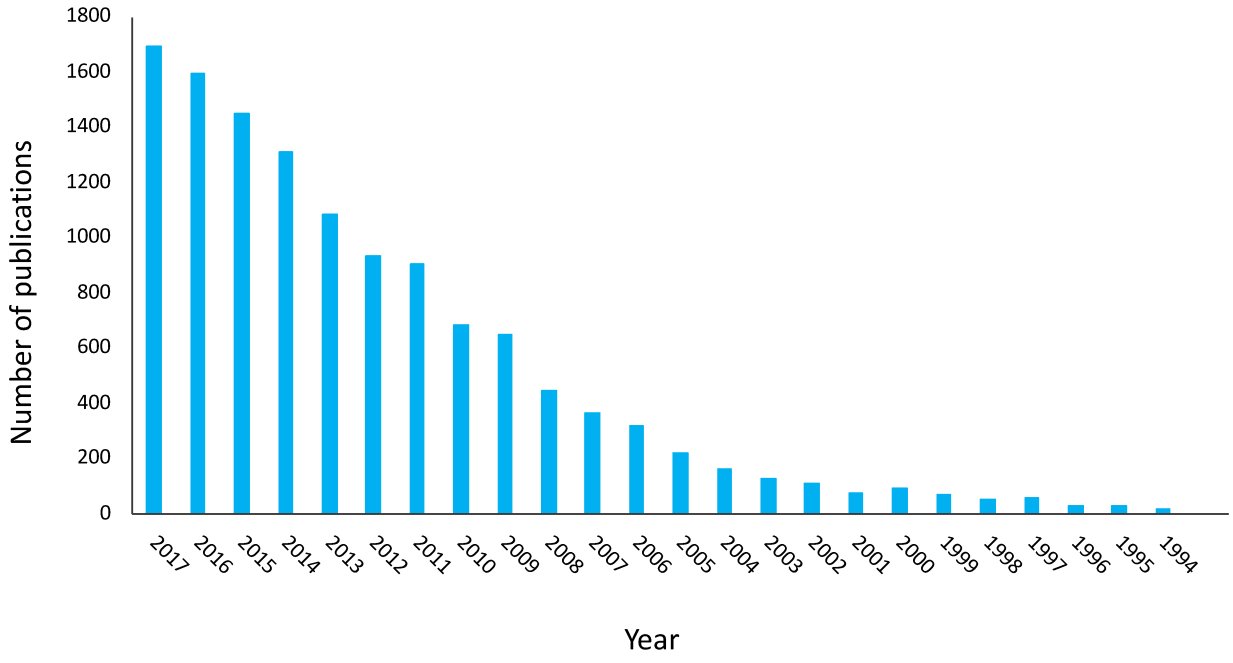


Figure 3.2 Trend with time of publications concerning aptamers (Source: Web of Science).

Despite many aptamers were reported until now in the literature, only few of them have found application. The most studied is, without doubt, the thrombin aptamer. Thrombin's aptamer is the most commonly used model to demonstrate the proof-of-concept of an aptamer-based affinity assay.[11] Thrombin is involved in various diseases, it plays an important role in physiological and

pathological coagulation. The first aptamer described for this target was a 15-mer DNA oligonucleotide (5'-GGT TGG TGT GGT TGG-3'), also another aptamer has been described with a different binding site to the target protein.[12] More than one hundred assays based on these aptamers binding thrombin were reported. Aptamers against many other targets have also been developed; among them, aptamers against tetracycline,[13] streptomycin,[14] PSA (Prostate Specific Antigen),[15] and Lysteria monocytogenes[16] are same examples, demonstrating the versatility of these analytical tools.

3.1.1 Aptamers versus antibodies

Aptamers show some interesting advantages over antibodies. In fact, as shown in Table 3.1, they have higher thermal stability and their production is animal free and cost effective, as well as their selectivity for targets make them a good alternative to monoclonal antibodies.

Feature	Aptamers	Antibodies
Selection of target	Few limitations	Immunogenic target
Quality control	No batch to batch variation	Batch to batch variation
Chemical modification	Easy	Difficult
Thermal stability	High	Low
Shelf life	Years in dehydrated form	Limited
Production	In vitro	In vivo

Table 3.1 Comparison of aptamers and antibodies characteristics.

Antibodies are Y-shaped proteins, they are naturally present in our immune system to protect us from pathogens. The capability of antibodies to interact with antigens is given by the variable domain in the light chains.

Antibodies used for research and diagnostic purposes are produced in a lab animal (i.e. rabbit or goat). Within a few weeks, the animal's immune system will produce high levels of antibodies specific for the antigen. These antibodies can be harvested in an antiserum, which is whole serum

collected from animals exposed to an antigen. Because most antigens are complex structures with multiple epitopes, they result in the production of multiple antibodies in the lab animal. These are called polyclonal antibodies (pAbs), (see Figure 3.3). Antiserum obtained from animals contains not only antibodies against the antigen artificially introduced in the laboratory, but it also contains antibodies from other antigens to which the animal has been exposed during its life. Consequently, a purification step is needed in order to remove other antibodies before using the antibodies, for research or diagnostic assays.

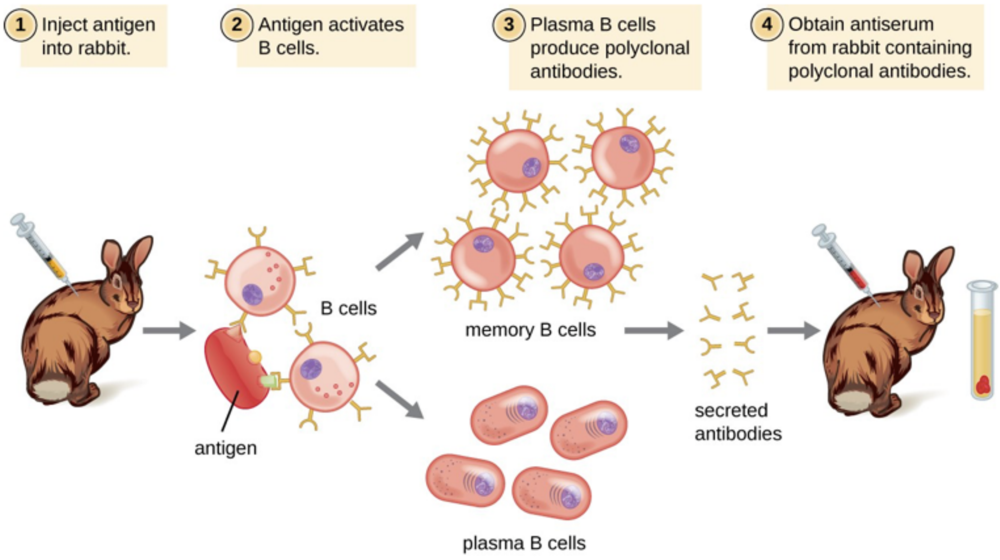


Figure 3.3 Procedure for the production of polyclonal antibodies. (Copyright: Rice University, License: http://creativecommons.org/licenses/by/4.0/).

However, some assays require the use of antibodies with very high specificity and affinity, provided by monoclonal antibodies (mAbs), which are able to bind with high affinity a single epitope. Unlike polyclonal antibodies, monoclonal antibodies are produced in vitro using tissue-culture techniques. They are produced by immunizing animals, often mouses, multiple times with a specific antigen. B cells from the spleen of the immunized animal are then removed and fused with immortal, cancerous B cells called myeloma cells, to yield hybridoma cells. All cells are then placed in a selective medium that allows their grow. The hybridoma cells are then screened for the desired mAb (see Figure 3.4). This is a very expensive and time-consuming process.

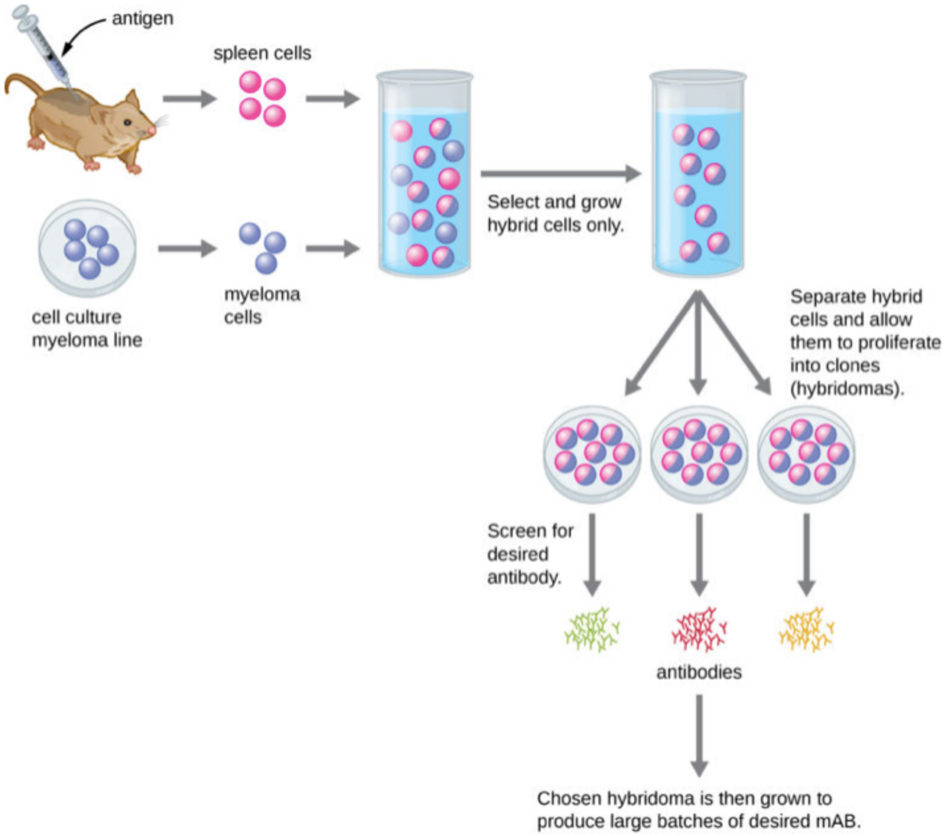


Figure 3.4 Procedure for the production of monoclonal antibodies. (Copyright: Rice University, License: http://creativecommons.org/licenses/by/4.0/).

Some of the drawbacks of antibodies are the batch-to-batch variability, their production is expensive and time consuming and they are sensitive to pH and buffer conditions.

Despite the advantages of using aptamers and the interest aroused in the academic field, the success of aptamers and their commercial potential is undervalued. A market deeply rooted in the use of antibodies prevent the economic evolution towards new products based on aptamers.[17] Table 3.2 gives an overview of companies already involved in aptamer commercialization.[18]

Company	Location	Web site	Founded
Alpha Diagnostic International	United States	http://www.4adi.com	1993
AMBiotech	United States	http://am- biotech.com	2009
AptaBiosciences	Singapore	http://www.aptabiosciences.com	2013
Apta Biotherapeutics	South Korea	http://www.aptabio.com	2013
Aptagen	United States	http://www.aptagen.com	2004
Aptahem	Sweden	http://aptahem.com	2014
Aptamer Group	United Kingdom	http://www.aptamergroup.co.uk	2008
Aptamatrix	United States	http://www.aptamatrix.com	2003
Apterna	United Kingdom	http://apterna.com	2011
Aptitude Medical	United States	http://www.aptitudemedical.com	2011
Aptus Biotech	Spain	http://www.aptusbiotech.com	2010
Aptamer Sciences	South Korea	http://www.aptsci.com	2011
Basepair Technologies	United States	http://www.basepairbio.com	2011
Berlin Cures GmbH	Germany	http://berlincures.de	2014
CD Genomics	United States	http://www.cd-genomics.com	2004
Ice9Biotechnologies	United States	http://www.iceninebio.com	2009
Neoventures Biotechnology	Canada	http://www.neoventures.ca	2002
Novaptech	France	http://www.novaptech.com	2008
NOXXON Pharma Ag	Germany	http://www.noxxon.com	1998
Ribomic	Japan	http://www.ribomic.com	2003
SomaLogic	United States	http://www.somalogic.com	2000

Table 3.2 Companies involved in aptamer commercialization.[18]

3.1.2 Biosensor applications

Generally, biosensors are classified according to their transduction principle such as optical (including optical fibre and surface plasmon resonance biosensors), electrochemical (including voltammetric, amperometric, and impedance biosensors), and piezoelectric (including quartz crystal microbalance biosensors). Alternatively, their classification is based on their recognition element, so that they are named immunosensors, aptasensors, genosensors, and enzymatic biosensors when the biological sensing element are antibodies, aptamers, nucleic acids, and enzymes, respectively.[19] As described in Figure 3.5, biosensors exploit the sensitivity of transducers combined with the high specificity of biological recognition elements, which are able to interact selectively with analytes. The impact of biosensing is gaining importance in all sectors, such as pharmaceutical, environmental and food fields.[20,21,22] In fact, biosensors can be considered very versatile and powerful tools, in view of their low-cost and compatibility with portable and compact instrumentation, as well as for their easy and rapid use.

Electrochemical biosensing platforms combine covering chemistry, material science, biological science, and medical industries. A significant example of a successful electrochemical biosensing is the glucose sensor, commercially known also as “The Lancet System” or “finger-prick test”. It allows the blood glucose concentrations to be tested by measuring a current directly proportional to the blood glucose level. In the last years, a lot of progress has been made; for instance, a continuous glucose monitoring system has been introduced, that allows a better control of a patient’s blood sugar levels. This technology is based on implantable transmitters detecting blood sugar levels directly in the body.

With the same purpose, wearable electrochemical sensors have been developed which integrate, in a non-invasive and non-obtrusive way, sensing systems suitable for various monitoring applications directly onto either textile materials or the patient epidermis.[23] These innovations point out that it is possible to develop technologies that can have real repercussions on the market.

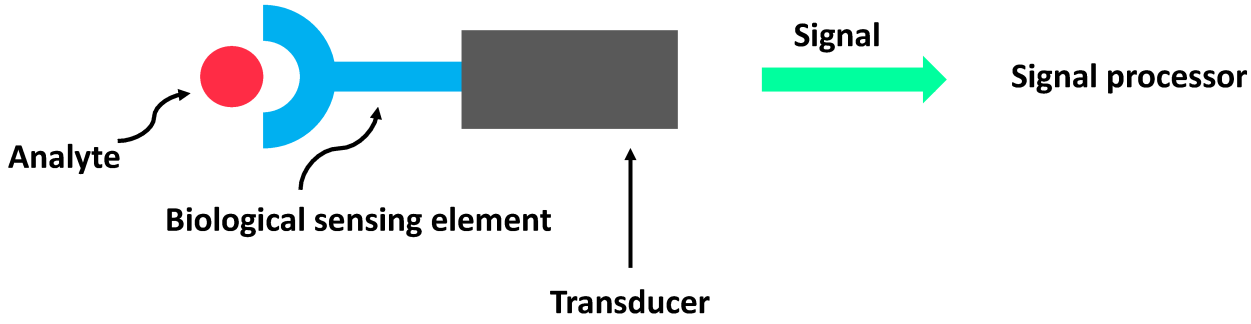


Figure 3.5 Schematic diagram of a biosensor.

3.2 SELEX

SELEX is the acronym for Systematic Evolution of Ligands by EXponential enrichment. It is a process, based on combinatorial chemistry, that was firstly described in 1990 by Tuerk and Gold.[24] It is characterized by the synthesis and simultaneous screening of large libraries of related, but structurally distinct compounds to identify and isolate functional molecules.[25] Very complex libraries of random oligonucleotides with about 10^{15} different molecules can be produced by chemical synthesis and screened in parallel for a particular functionality. The selection starts with the interaction between the library, with predesigned primer-binding domains for later PCR amplification, and the target of interest; unbound sequences are washed, while sequences displaying affinity for the target are collected and amplified through PCR for next rounds. This process is repeated until the library converges onto a collection of sequences that really display affinity for the target of interest. A schematic description of SELEX is reported in Figure 3.6.

Tuerk and Gold described for the first time this process isolating RNA aptamer sequences against bacteriophage T4 DNA polymerase.[26] Later, Ellington and Szostak, reported the successful selection of RNA sequences able to bind specifically a variety of organic dyes.[27]

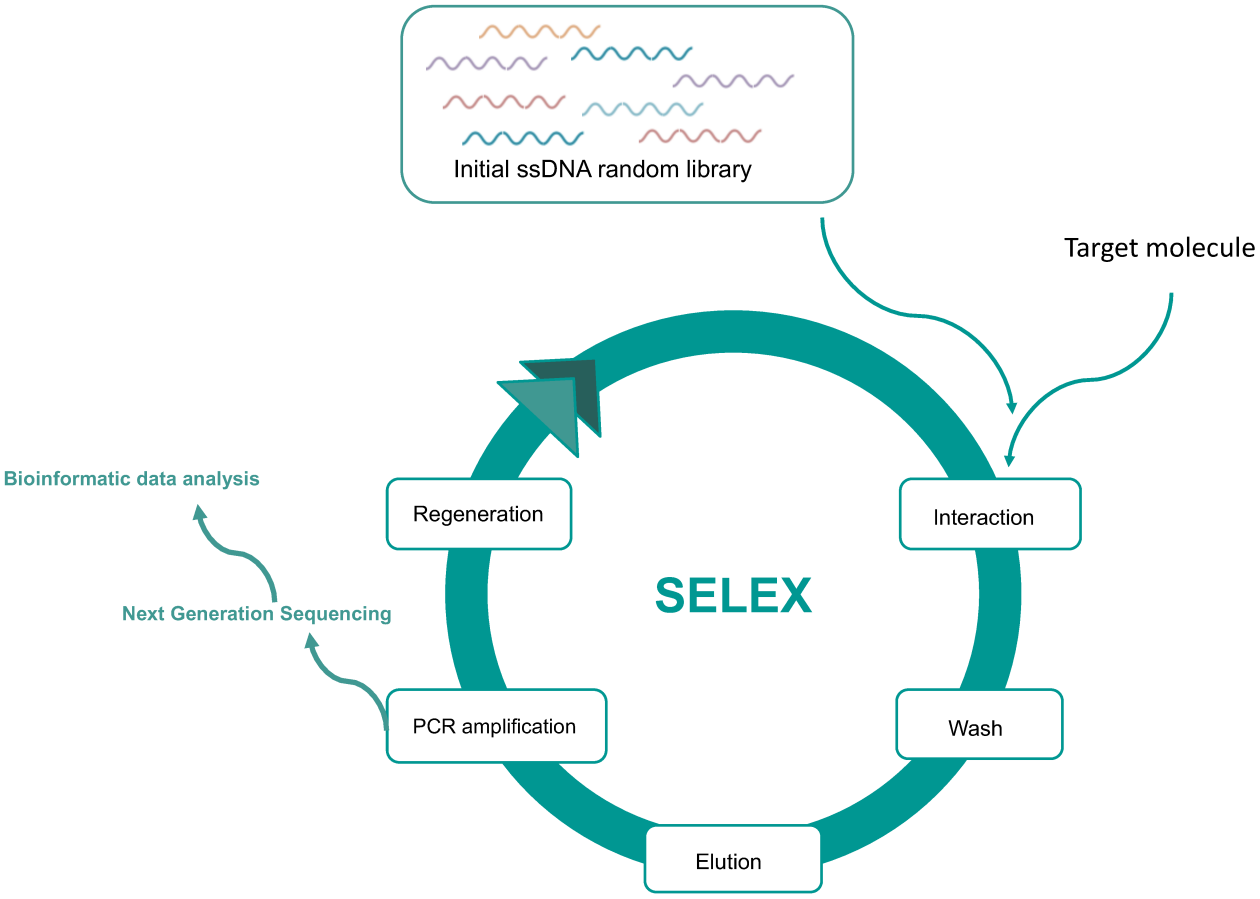


Figure 3.6 Schematic diagram of the SELEX process steps.

3.3 DNA stability and behavior in deep eutectic solvents

DNA is usually employed in aqueous solutions, where its helix structure can be destroyed by non-physiological temperatures, pHs and ionic strengths. In addition, degradation processes promoted by nucleases can lead to its chemical instability.[28] This instability can be a drawback in biotechnology based on nucleic acids. Furthermore, the fact that very small water volumes vaporize immediately under open-air conditions or at high enough temperatures can be a further limitation in the development of DNA-based nanotechnologies, where really small volumes are usually employed. Consequently, the use of solvents without these limitations could open up new possibilities in the development of DNA-based devices. With this view, the possibility of using deep eutectic solvents (DESs) for biotechnological applications appears to be of unquestionable interest.[29]

In particular, it has been reported that DESs based on the choline ion ensure long-term stability of biomolecules like DNA and proteins.[30] It was proved recently that DNA can form a stable duplex in reline.[28]

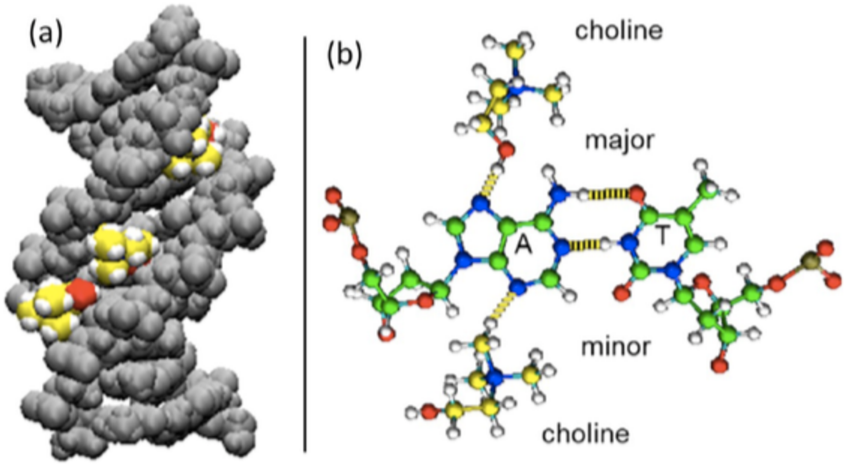


Figure 3.7 (a) Choline ions solvating the minor groove of A-T base pairs of duplex DNA. (b) atomic scale representation of the DNA-Choline bounds. (Hydrogen bonds are shown as yellow dotted lines).

The interaction between ionic solvents and DNA comes from the electrostatic attraction of organic cations and the DNA phosphate backbone, followed by hydrophobic and polar interactions between ionic liquids and the DNA major and minor groove.[31] In the case of choline chloride based DESs, choline ions interact with atoms belonging to all areas of DNA through multiple hydrogen bond networks, thus stabilizing DNA duplex more effectively than sodium ions present in aqueous buffers. As to DNA stability, cations play a more important role than anions, because cationic species are required to reduce the repulsive forces between phosphate groups of DNA strands, while anions just interact with the bases through hydrogen bonds.[32]

As shown in Figure 3.7, choline ions have a high affinity for the A–T base pair minor groove due to the narrower width and more electrostatically polar environment of this groove relative to the major groove.

On the basis of all these considerations, together with the ethaline's ability to extract gluten, the development of aptamers in this medium could represent an innovative and interesting approach for the development of more effective biosensors.

3.4 Aim of this work: DES-SELEX

We decided to select aptamers targeting gluten proteins in the DES, called ethaline, which proved to be in our previous work very effective for gluten extraction (see Chapter 2).

In particular, we focused our activity on the development of aptamers able to recognize extracted proteins directly in DES media without lowering sensitivity.

Although DESs allow the creation of functional DNA structures of greater complexity and different from those obtained in water, thus suggesting that they may favor the folding of aptamers into functional shapes, no report is until now available concerning aptamer selection in DESs.

During the description of the work, for the sake of comparison, we will refer to a procedure performed to select aptamers against gluten in aqueous buffer reported in the literature.[33]

3.5 Experimental

3.5.1 Reagents and Apparatus

Unmodified and 5'-tagged (biotin) aptamers were obtained from Laboratorios CONDA (Madrid, Spain). Peptide 33-mer was obtained from Biomedal (Sevilla, Spain). All sequences are summarized in Table 3.3. Gliadin standard from Prolamin Working Group (PWG) was acquired to R-Biopharm AG (Germany). Ethaline was supplied by Scionix Ltd. (London, UK) and employed as received. All aqueous solutions were prepared with water purified with a MIlliQ system (Millipore, Spain). Salts for buffer solutions, Tween-20, Bovine Serum Albumin (BSA), 1 M TRIS/HCl pH 7.4, Phospate Buffered Saline (PBS) 10× and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA solution were obtained from Sigma-Aldrich (Spain). Immolase DNA polymerase, buffer, and magnesium salt for PCR were obtained from Bioline (UK). The 5× TBE (0.45 M Tris-Borate and 0.01 M EDTA, pH 8.3) was acquired from 5 Prime (Maryland), and the 6× DNA gel loading buffer was acquired from Novagen (San Diego). Dynabeads™ MyOne™ Streptavidin C1 (strep-MPs), Dynabeads® M-280 Tosylactivated, DNA and Protein Lo-bind Eppendorf tubes, Eppendorf ep T.I.P.S PCR and Streptavidin-peroxidase conjugate (strep-HRP) were obtained from Thermo Fisher Scientific. Electrochemical measurements were performed with a computer-controlled μ-AutoLab type II potentiostat with Nova 2.1 software (EcoChemie, The Netherlands). Thermomixer (Eppendorf Iberica, Spain) and the magnet (DynaMag-2) for magnetic separation were purchased from Life Technologies (Madrid, Spain). PCR was carried out in a GeneAmp PCR system 9700

(Applied Biosystems, Life Technologies). ROX Reference Dye was obtained from Invitrogen, EvaGreen (Biotium) was acquired from VWR (Spain). Disposable screen-printed carbon electrochemical cells were purchased from Metrohm-Dropsens (Spain).

3.5.2 SELEX procedure

The immunotoxic peptide, 33-mer, was chosen as the target for our aptamer selection. This choice was suggested by the fact that it is a highly hydrophobic peptide identified as one of the most immunogenic fragments from α2-gliadin. It was modified with a biotin tag in its C-terminus and immobilized onto streptavidin modified magnetic particles (MPs) following the protocol below.

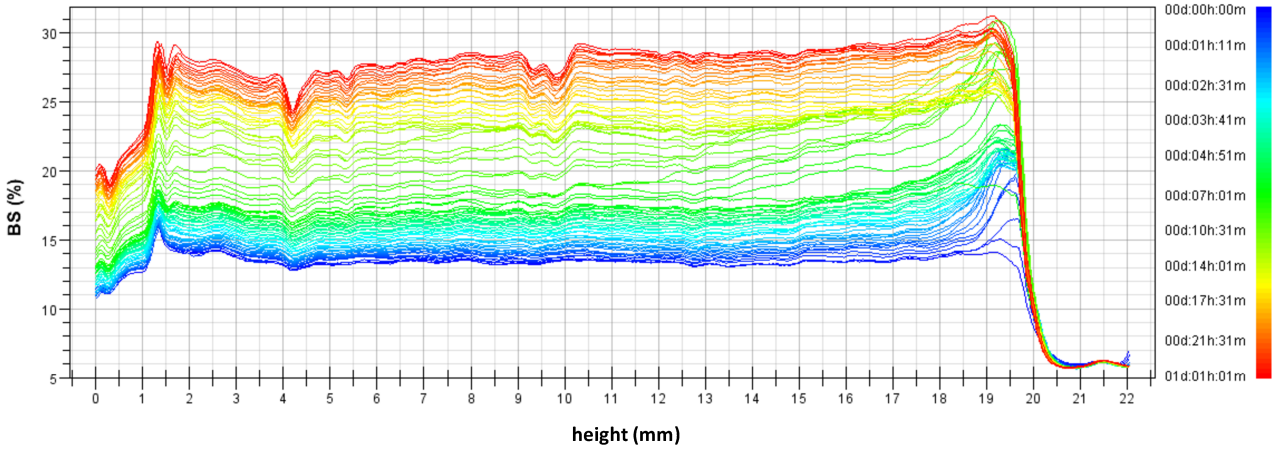
50 µL of strep-MPs were washed twice with 1 mL PBS (Phosphate Buffered Saline) + 0.01% Tween-20 and resuspended in PBS containing 2 µM of biotinylated 33-mer. Streptavidin-biotin interaction was conducted for 30 min at 30 °C under continuous shaking in a Thermomixer. Subsequently, the magnetic beads were washed with PBS + 0.01% Tween-20 and blocked with 500 µM biotin in PBS + 0.01% Tween-20 for 30 min. After two washes, magnetic particles were reconstituted in 500 µL of Ethaline and were ready to be used in SELEX.

The colloidal stability of the modified-MPs suspension in both aqueous buffer and ethaline was monitored by multiple light scattering (see Figure 3.8). Measurements were carried out with a Turbiscan Lab equipment (Formulaction, France) coupled to an aging station of the same brand, which allows automatic analysis over a specific time. Specifically, light backscattering measurements were made along the height profile of the cell at 30 °C. A total measurement time of 24 hours was adopted, with profiles recorded every 10 min during the first 6 h, and every 30 min for the remaining hours.

The stability of the magnetic particle suspension in aqueous buffer and in ethaline was different, as shown in Figure 3.8. Magnetic particles modified with 33-mer and dispersed in ethaline remained in suspension for a longer time than in aqueous buffer, where they precipitated (speed of 0.06 mm/h) after an agglomeration phenomenon which was detected as an increase of light backscattering in the middle zone of the cell. In the deep eutectic solvent, a homogenous distribution of particles was instead observed throughout the entire measurement cell, which remained stable (minimum variation) for 24 h, with no precipitation. While relatively fast aggregation of MPs was observed in aqueous buffer, no aggregation was observed in DES. This

ability of DES to stabilize modified particles appears to be of great importance for their use over time.

(A)



(B)

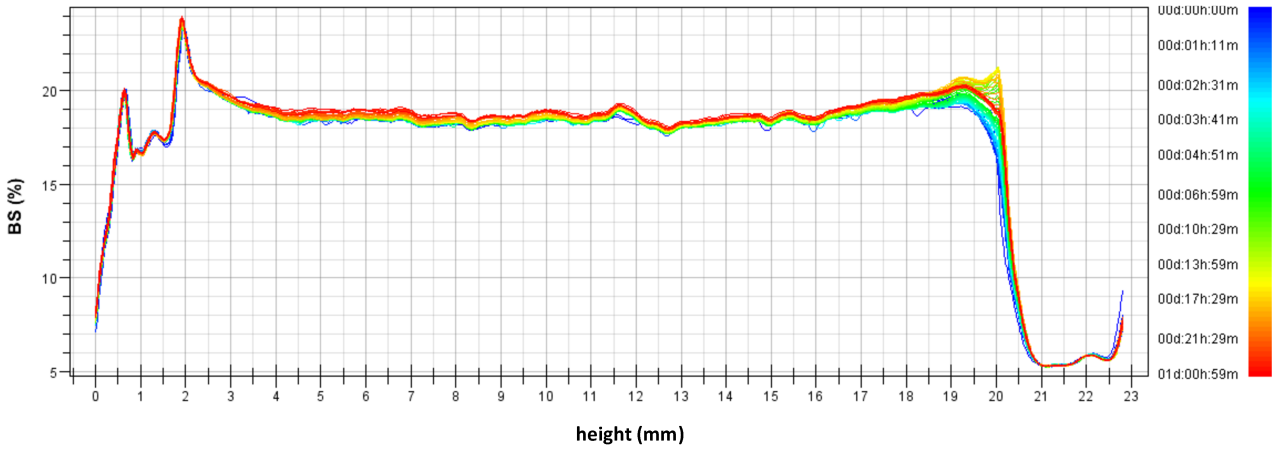


Figure 3.8 Back scattering versus sample height and time for the sedimentation of a 20 µg/mL suspension of 33-mer-modified microparticles in (A) aqueous buffer, (B) ethaline.

So, we proceeded with the first SELEX round that was performed by incubating 1 nmol of a starting library of 80-mer DNA oligonucleotides with a central 40-mer random region flanked by primer binding sequences (see Table 3.3), with 20 µg of microparticles with the immobilized peptide in ethaline (1 mL total volume); 0.1 nmol of tRNA were incorporated as a competitor, and the unspecific binding of oligonucleotides was minimized using BSA in ethaline solution. After interaction for 60 min the bound oligonucleotide fraction was magnetically separated and eluted with hot water. Recovered oligonucleotide sequences were then amplified by PCR with a biotinylated reverse primer; amplification products were entrapped onto streptavidin-modified

MPs and denatured with NaOH to prepare a new pool for further selection rounds. This process was repeated under progressively increased stringency by varying the interaction time from 60 to 30 min and the number of washing steps after interaction from 2 to 5 (see Table 3.4).

Description	Sequences
Biotinylated reverse primer	Biotin-CCA ACA GCT AGT TGA CAT CG
Reverse primer	CCA ACA GCT AGT TGA CAT CG
Forward primer	AGG GTT GAT AGG TTA AGA GC
Initial ssDNA library	AGG GTT GAT AGG TTA AGA GC - 40N - CGA TGT CAA CTA GCT GTT GG
Biotinylated 33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF – Lys - Biotin

Table 3.3 Nucleic acid sequences, written from 5' to 3', and peptide sequence used in this study.

3.5.2.1 SELEX - Interaction protocol

For the SELEX procedure we employed a random ssDNA library containing 40 randomized positions at the 1 µmol scale. In the first round of selection, 1 nmol of the library was heated at 98 °C for 5 min and cooled on ice, then the library was added to MPs modified with 33-mer. 1 µg/mL BSA was added to the selection mixture to avoid unspecific binding.

tRNA was also used as a competitor to make more stringent the selection. The [t-RNA]/[ssDNA] ratio was kept at 0.1.

The interaction step between ssDNA library and the peptide was carried out for 1h at room temperature in ethaline. After this step, magnetic separation was performed, and the supernatant was discarded. MPs were washed as reported in Table 4. Then, the ssDNA-peptide complex was eluted with 30 µL of purified water at 95 °C for 15 min.

Round	Counter selection	[33-mer] / [DNA]	[tRNA] / [DNA]	$t_{incubation}$ /min	Washings
1		10	0.1	60	2*
2		10	0.1	60	2*
3	**yes**	10	0.1	60	2**
4		10	0.1	30	2***
5		10	0.1	30	5****
6		10	0.1	30	5*****

Table 3.4 Summary of SELEX conditions adopted.

* 2 washings with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20

** 1 washing with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 + 1 with purified water

*** 1 washing with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 + 1 with 1 NaCl 1M

**** 1 washing with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 + 2 with NaCl + 2 with purified water

***** 1 washing with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 + 3 with NaCl + 1 with purified water

3.5.2.2 SELEX - Amplification Protocol

After DNA elution, winner aptamers were amplified by polymerase chain reaction (PCR), after a 1000 fold dilution. Each PCR vial contained 2 µL of template (eluate), 1 µM of each primer, 0.2 mM dNTP, 3 mM Mg^{2+}, 1× PCR buffer and 1 U hot-start immolase^{TM} DNA polymerase. The mixture was amplified with the following protocol, which is summarized in Figure 3.9: 95 °C 10 min (enzyme activation), 16 cycles of 45 s at 94 °C, 45 s at 57 °C, 45 s at 72 °C (denaturation and annealing steps), 72 °C for 10 min (extension), (see Figure 3.9). After each PCR a 2% agarose gel electrophoresis using a marker of DNA sizes of 20 base pairs was performed to ensure that this protocol generated a product of the appropriate size.

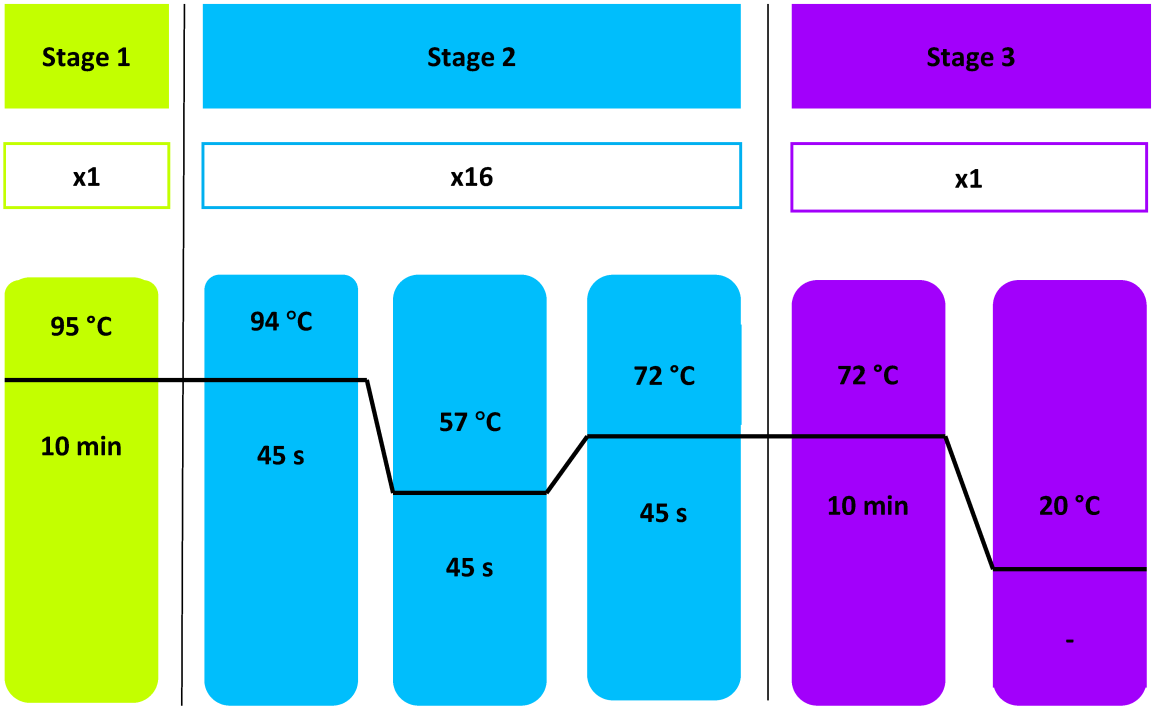


Figure 3.9 Temperature profile of PCR.

3.5.2.3 SELEX - Agarose gel electrophoresis protocol

PCR product was determined by 2% agarose gel using a low size DNA ladder. Samples and a low size DNA ladder were loaded on the agarose gel with 1×TBE (Tris Borate EDTA) as a running buffer. Electrophoresis was carried out for about 30 min applying a potential across the electrodes of 120 V. Amplified sequences were visualized with SimplySafe in an UV transilluminator.

Moreover, we verified that possible small amount of ethaline still present after washes didn't interfere with the PCR procedure. With this purpose, a PCR of the starting library was performed in the presence of 0.1, 1 and 10% of ethaline. As it can be seen from Figure 3.10, low amounts of ethaline do not interfere with the amplification process. In fact, the product of the expected length is obtained without artifacts. Only in the presence of consistent ethaline concentrations, such as 10%, the amplification is inhibited because there is no product of PCR. In our case, considering the washings carried out and reported in Table 4 ethaline amounts exceeding 1% are not expected.

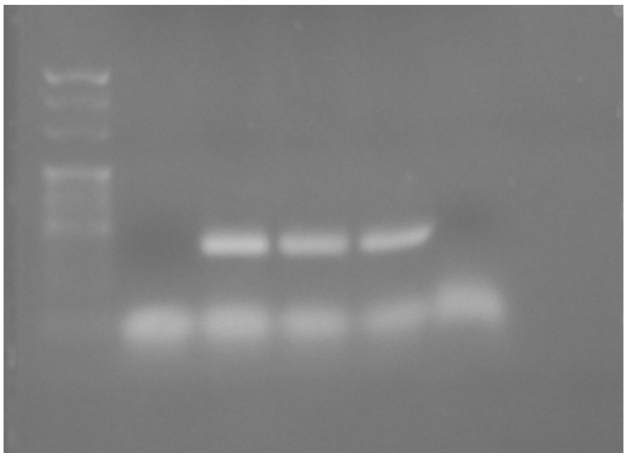


Figure 3.10 Agarose gel of PCR containing growing quantities of ethaline. Column 1: size standard, column 2: blank, column 3: library with 0.1% of ethaline, column 4: library with 1% of ethaline, column 5: library with 10% of ethaline.

3.5.2.4 SELEX - Regeneration protocol

Double stranded DNA was magnetically separated using biotinylated MPs. Complementary strand recovery from streptavidin beads was performed by elution with NaOH. All subsequent rounds were performed with 200 pmol of DNA. During the third round we performed a counter selection employing streptavidin MPs modified only with biotin.

3.5.3 Enrichment assay

The in vitro selection process can be monitored in terms of ssDNA quantities that are eluted during different SELEX rounds. To evaluate SELEX progression and the effect of the ethaline medium on the selection efficiency, we compared the enrichment rate of sequential ssDNA pools with affinity towards the immunotoxic peptide. An aliquot of ssDNA from each round was PCR amplified. The forward strand was separated from the biotinylated one by entrapment on strep-MPs and subsequent denaturation with NaOH. The final concentration was determined spectrophotometrically at 260 nm. The binding assay was carried out equilibrating equimolar amounts of ssDNA from each cycle and 33-mer-modified MPs for 30 min in ethaline in a mixer wheel

at room temperature, followed by magnetic separation of the unbound fraction (supernatant) using a suitable magnet. After one washing step with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ + 0.01% Tween-20 and two with purified water, the bound fraction was eluted with 30 µL of purified water at 95 °C for 15 min. It was then collected and measured at 260 nm with an Agilent Cary 60 UV-Vis equipped with a micro-volume TrayCell designed for the UV/Vis analysis of DNA. The results were compared with those previously obtained by SELEX in aqueous buffer. Since the first rounds of selection in ethaline, an enrichment of the initial library significantly faster than that obtained in aqueous medium became evident, with a roughly 3-fold increase with respect to the aqueous selection after six rounds as shown in Figure 3.11. The faster enrichment could be probably ascribed to the higher viscosity of ethaline which can impose more stringent conditions during selection or alternatively to the potential ability of DES to alter the secondary structure of nucleic acids, thus leading to an additional structural selection.

The enrichment after six rounds of selection in ethaline was clearly greater than after 10 rounds in water.

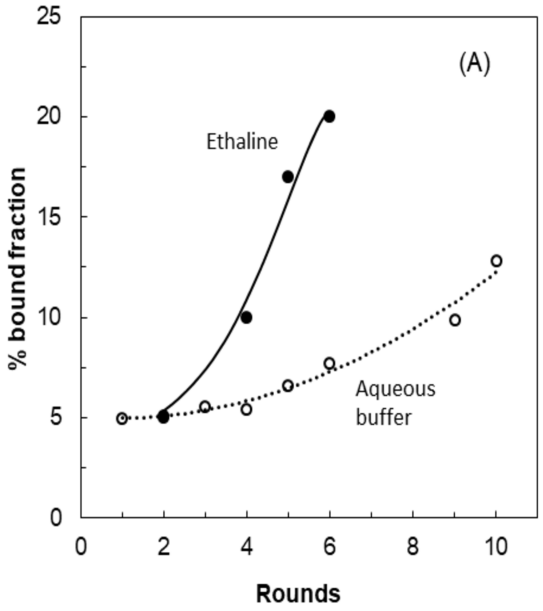


Figure 3.11 Enrichment assay of oligonucleotides bound to the 33-mer-modified MPs throughout the SELEX rounds in ethaline versus aqueous buffer.

3.5.4 Remelting study

The 7900HT Fast Real-Time PCR System (Thermo Fischer Scientific) was employed to perform remelting curves. Starting from amplified samples of each SELEX round EvaGreen and ROX Reference Dye were added to the final concentration of 1X. Melting curve analysis was performed at 95°C for 15 s, at 50° C for 30s with the last step at 95° C for 30 seconds (see Figure 3.12).

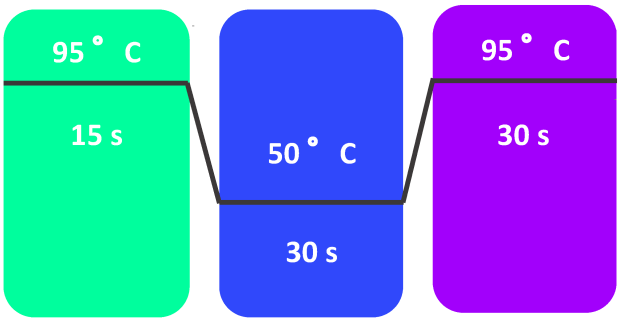


Figure 3.12 Temperature profile of remelting procedure.

Remelting curves are good characteristic indicator for the diversity changing through SELEX evolution.[32] Concomitantly to an increase of diversity, a decrease in melting temperature is usually detected. In our analysis a biphasic sequences distribution is observed after fourth round (see Figure 3.13), but unexpectedly the growing remelting peak appeared at a temperature (T_m =75 °C) lower than the remelting peak in the original library (T_m = 81 °C).

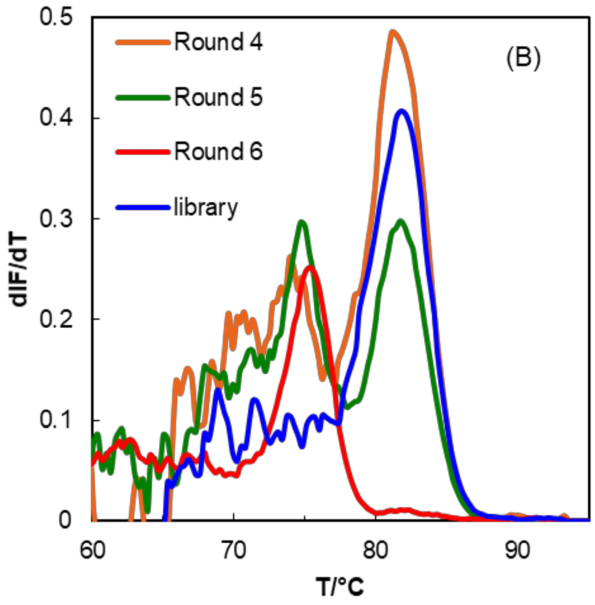


Figure 3.13 Remelting curves of the initial library and pools after the specified selection rounds in ethaline.

This is not compatible with the usual PCR artifacts arising from cross-hybridization of amplified library members, which once extended by polymerase give by-products longer than those expected and detrimental to the enrichment process.

Agarose gel electrophoresis analysis conducted during the SELEX process revealed that two different sets of products appeared after the fourth cycle, the expected 80-mer and shorter products (see Figure 3.14). Despite additional selection rounds were performed with only oligonucleotides of the expected length, purified by gel electrophoresis, shorter products continued to increase, and they became the only appearing species after the sixth round of selection. The presence of shortened sequences with lower melting temperature, may stem from template shifting during amplification pushed by the stabilization of some secondary structures in DES. These short-sequences were not observed when the same primers were employed in the selection of anti-33-mer aptamers in aqueous buffer. Although the presence of such sequences was not detrimental for the enrichment, we chose to analyze ssDNA surviving after the fifth cycle by high-throughput sequencing using the Illumina platform.

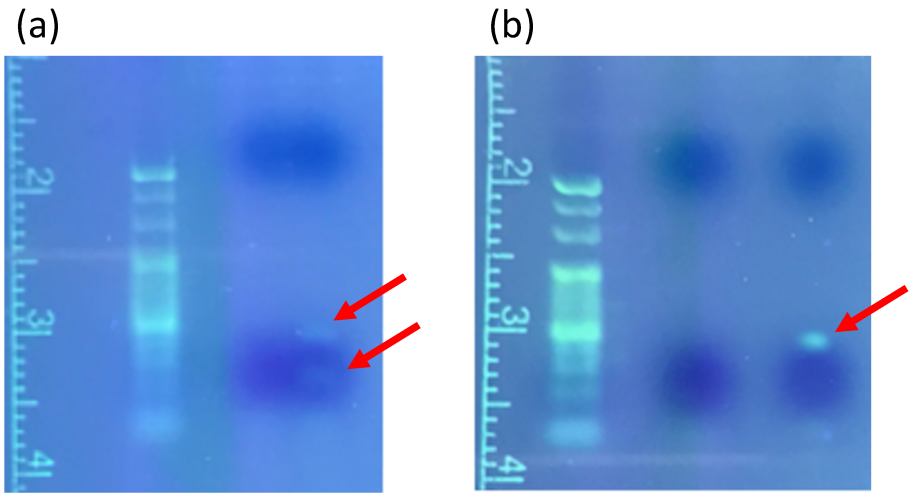


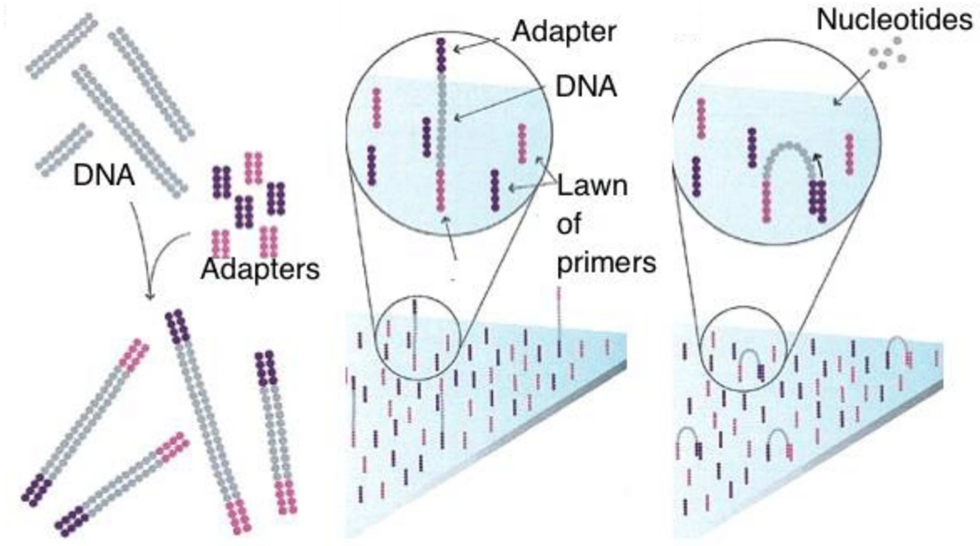
Figure 3.14 Typical example of gel electrophoresis of SELEX PCR products. DNA ladder and PCR product consisting of the expected 80-mer and shorter products indicated by the red arrows (a), DNA ladder and PCR product consisting only of the expected 80-mer indicated by the red arrow (b).

3.5.5 Illumina sequencing and data analysis

The sample from round 5 was sequenced exploiting Illumina Sequencing Technology (performed by IGA Technology Services, Udine), then the sequencing result was analyzed using Galaxy web platform and AptaSUITE software.

Illumina sequencing is a technique aimed at determining the exact sequence of a DNA, also known as DNA sequencing.

Illumina sequencing technology exploits clonal array formation and proprietary reversible terminator technology for rapid and accurate large-scale sequencing. The first step consists in the ligations of adapters to both ends of DNA fragments that need to be sequenced. Then fragments are attached to the flow cell surface. Then, after the addition of nucleotides and polymerase enzyme a solid phase bridge amplification starts, so that fragments become double stranded. Afterward, a denaturation step takes place which leaves on the surface single-stranded templates. In this way, several millions of DNA clusters are generated. The first sequencing cycle begins adding labeled nucleotides. After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified. The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time. A scheme of the procedure is reported in Figure 3.15.



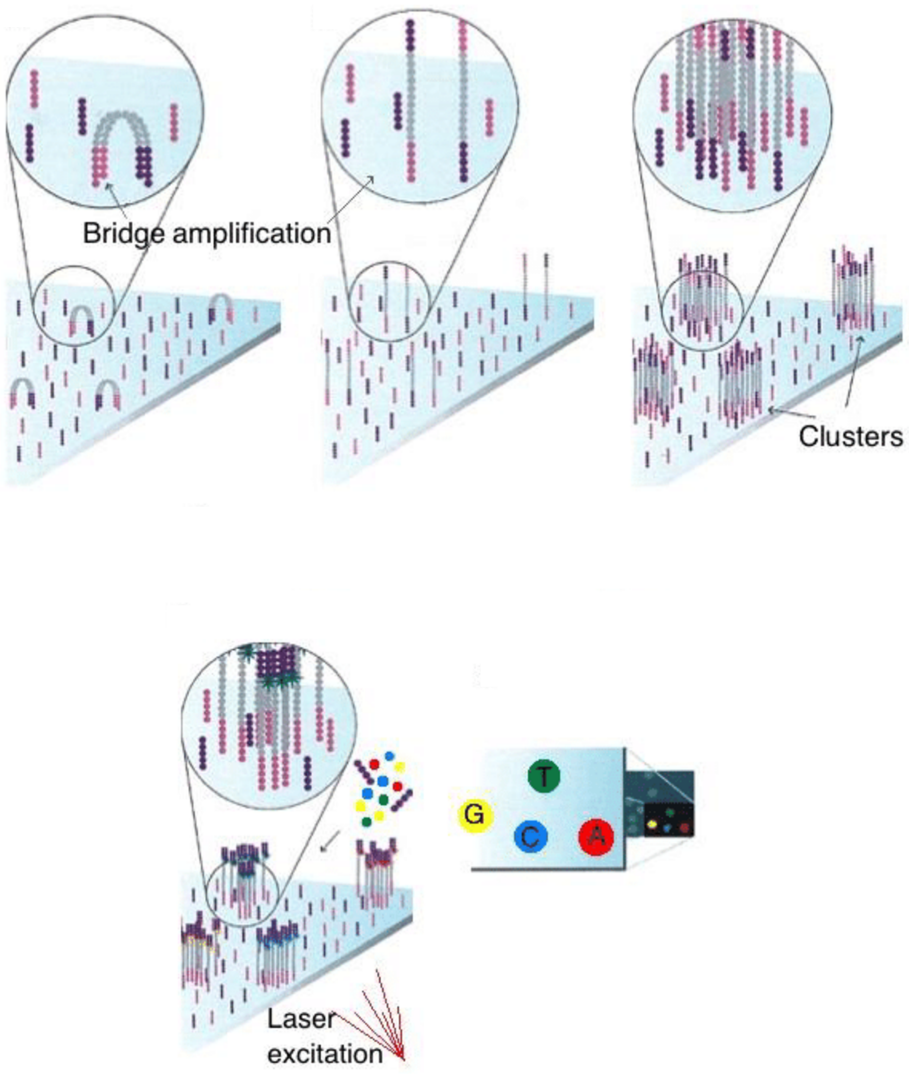


Figure 3.15 Illumina sequencing scheme.

Data obtained after Illumina sequencing were analyzed by the Galaxy web platform, using the public server at http://usegalaxy.org. Moreover, we also employed AptaSUITE, a graphical user interface for the efficient analysis of HT-SELEX data.[35]

3.5.5.1 Web-based Bioinformatic Analysis of Aptamer High-throughput Sequencing Data

Sequencing of the fifth round gave more than 1.5 million of reads. This large dataset needs a sophisticated aptamer bioinformatics method in order to lower the hundreds of millions of aptamers sequences to a select few candidates to test experimentally. The Galaxy Project webserver, originally created to analyze genomic high-throughput sequencing (HTS) data, is a collection of bioinformatic tools that are powerful, flexible, dynamic, and easy to use (see Figure 3.16). It is free and accessible from any web browser. However, HTS data analysis requires a wide knowledge of the tools present in Galaxy. So, as reference, we used a workflow reported in literature.[36] The first step was the removal of adapter sequences and constant region sequences and collapsing identical reads. For each sequence, the read count (abundance) was reported. The analyzed data were then downloaded and used to identify the aptamer sequences to be tested, analyzing motif repetition.

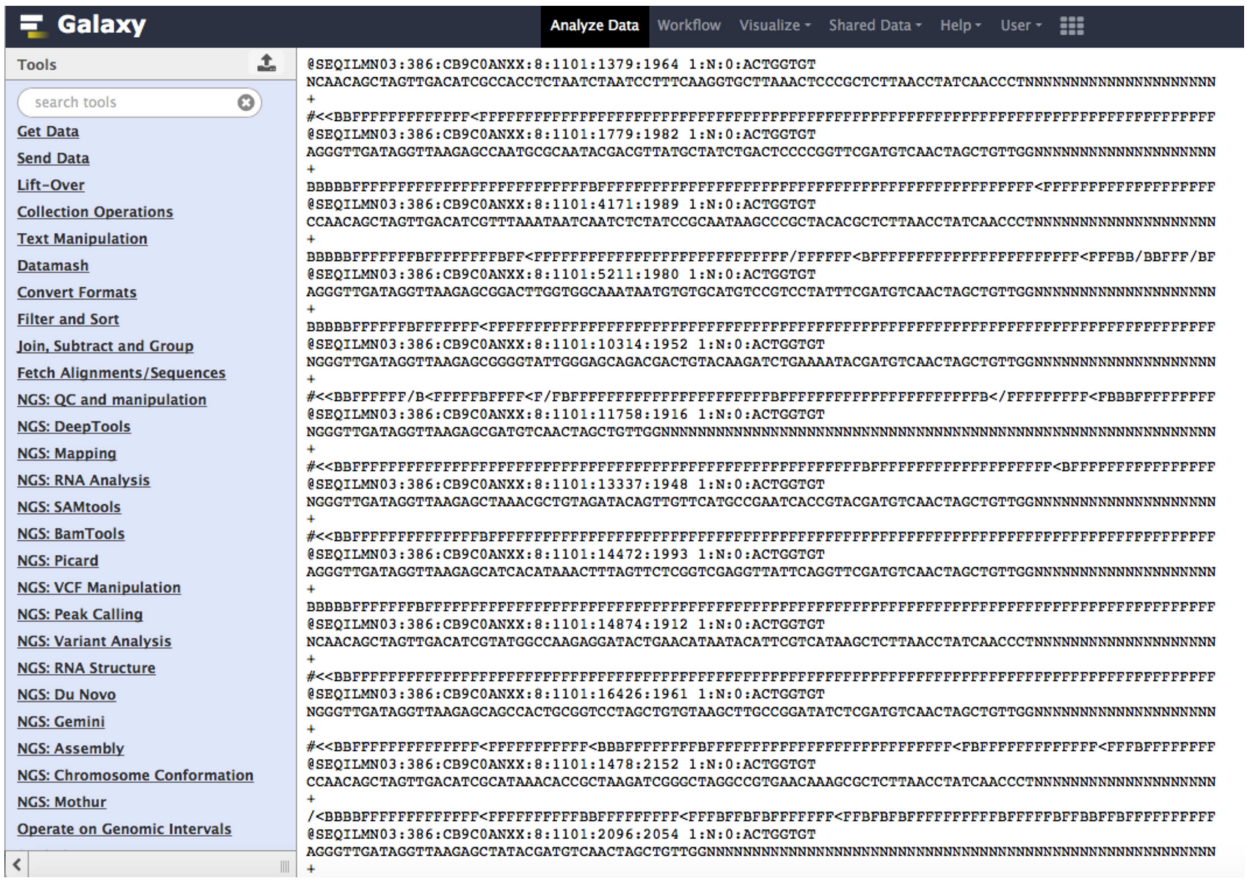


Figure 3.16 Galaxy platform.

In detail, we created a Galaxy access and uploaded the FASTQ file which was filtered for the quality of the reads and converted to FASTA format. Primers were removed in both senses (5' – 3') and reverse complements created. Sequences were filtered by length (40 or 20 ± 2 bases). Finally, with collapse tool, identical sequences were merged.

3.5.5.2 Bioinformatic Analysis of Aptamer High-throughput Sequencing Data using AptaSUITE software

AptaSUITE is a platform of multiple algorithms designed for the identification of aptamer candidate sequences and the analysis of the SELEX process. We followed the instruction provided by the relevant software, as shown in Figure 3.17, that guided us through the process step by step.

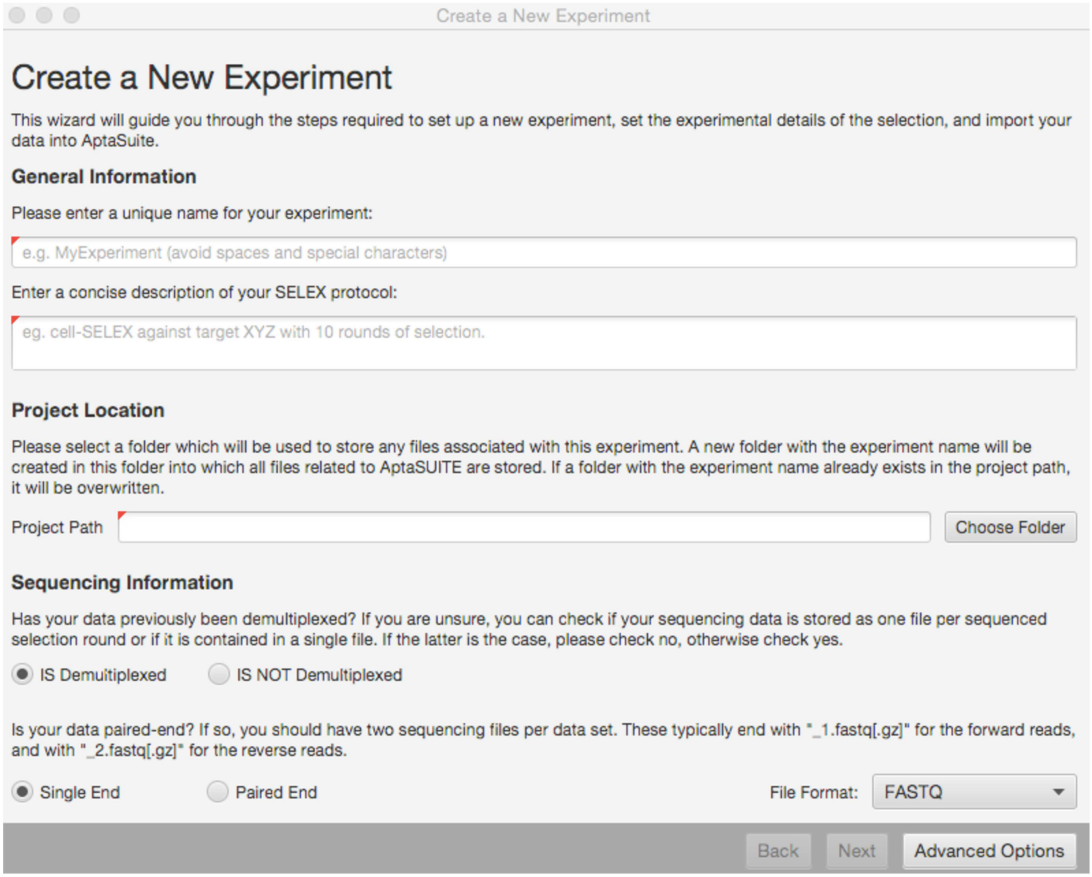


Figure 3.17 AptaSUITE software.

At the end of the analysis process, we compared the obtained results with those of the Galaxy platform.

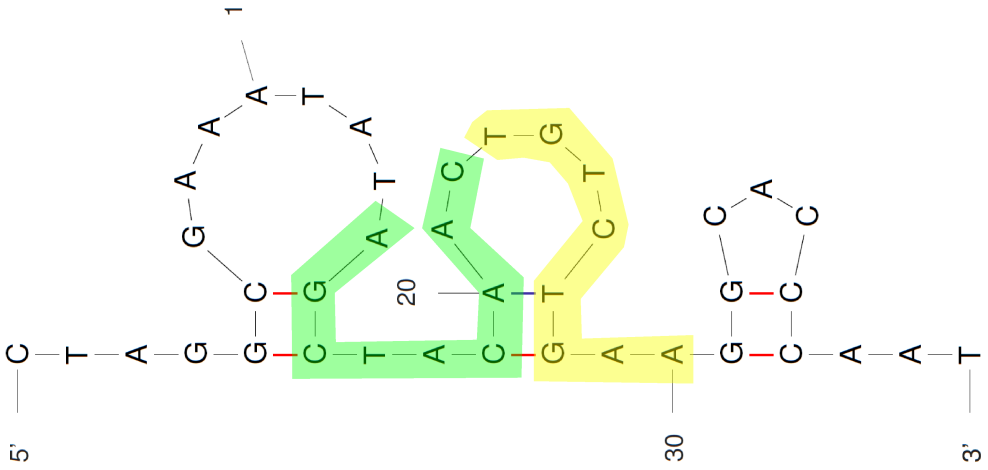
3.5.5.3 Bioinformatic analysis output

We obtained 829.782 unique sequences, without errors in primers, with a length of 40 nucleotides (nt). We also analyzed the sequencing output, isolating 20 nt sequences that were only 2.191. Interestingly, we found that the most abundant sequence, Gli1 (see Table 3.5), is the same one we identified as the most represented in a SELEX process against the 33-mer peptide in aqueous buffer.[33] By analysing the structural features of the potential aptamers, we identified two consensus sequences residing in hairpin structures. The first one is an 8 nt motif (TGTCTGAA) that appears in 518 different sequences, including Gli1 and Gli4, with the core 6 nt motif GTCTGA, in the loop of the hairpin structures, repeated in 1 % of the filtered sequences. The second one is a 9 nt motif (AGCTACAAC), which is common to 61 different sequences, including Gli1. The sequences Gli2D and Gli3D were selected as additional representatives of each group because they are characterized by the minimum free energy secondary structure in their respective group. In Table 5 Common motifs are highlighted in each sequence. Figure 3.18 shows the secondary structure of each aptamer selected and the corresponding value of ΔG. Secondary structure and ΔG were obtained using the Mfold web server for nucleic acid folding and hybridization prediction. For this analysis, 25 °C and the saline concentration of the selection buffer, 250 mM NaCl and 5 mM $MgCl_2$ were taken into account.

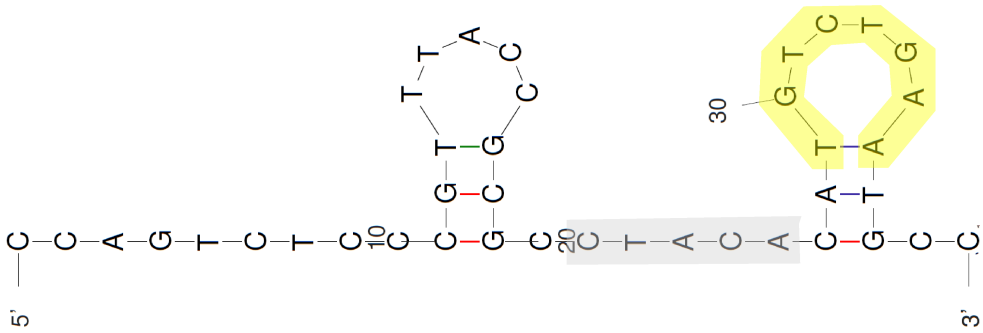
Name	Sequence
Gli1	CTAGGCGAAATATAGCTACAACTGTCTGAAGGCACCCAAT
Gli4	CCAGTCTCCCGTTTACCGCGCCTACACATGTCTGAATGCC
Gli3D	GCTGGGGCTTTACCTGCTGGGCATTAGCTACAACATGCT
Gli2D	TGATCGTTTTTATTGTGTCTGAACGTTTTGCAGTATGGAA

Table 3.5 Aptamer sequences obtained from massive sequencing of the fifth round.

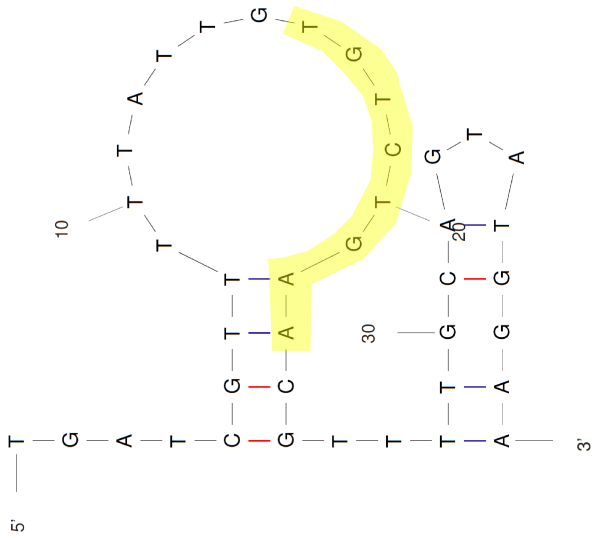
Gli1 (ΔG=-1.24 kcal/mol)



Gli4 (ΔG=-1.72 kcal/mol)



Gli2D (ΔG=-3.62 kcal/mol)



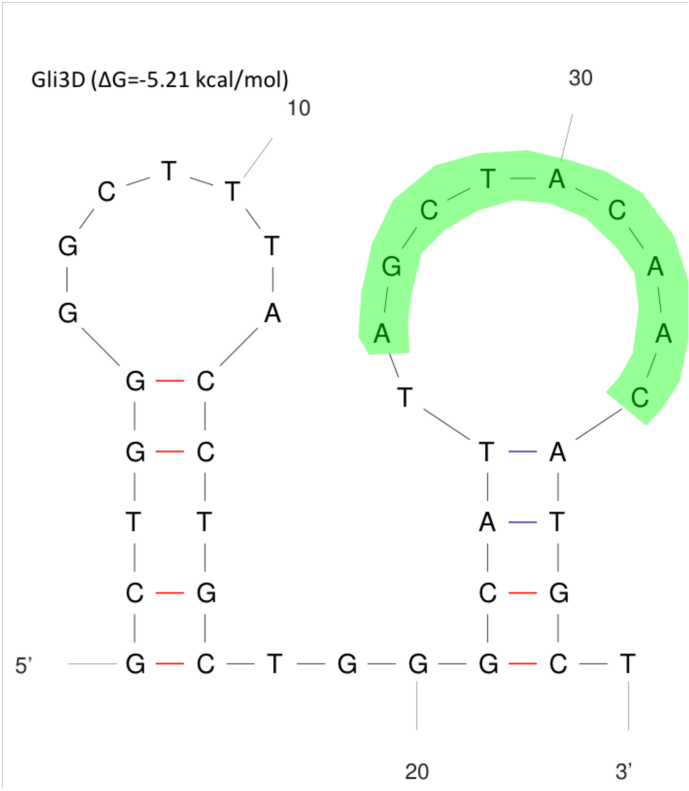


Figure 3.18 Secondary structure of selected aptamers.

3.6 Conclusions

The development of aptamers against gluten/gliadin is difficult to accomplish, as many researchers have openly recognized.[37] We took a step further by demonstrating the viability of SELEX in a green extraction solvent for the first time, thus providing a method for obtaining aptamers able to recognize non-soluble and poorly water-soluble molecules or species prone to aggregation in aqueous solutions.[38] This could have deep implications in the way that gluten analysis is currently performed, avoiding dilution and thus pushing down the limit of detection and outperforming the extraction yield. Among many advantages, ethaline demonstrated to stabilize modified magnetic particles and provided a faster selection than in aqueous media, reducing the number of selection cycles needed for achieving a similar enrichment factor from the starting DNA library. Moreover, the high viscosity of DESs may impose additional restrictions during the selection, which, added to the trapping of some folded states stabilized through a diffusion limited process propel the selection. Owing to its high efficiency while mimicking the intracellular media, the application of DES-SELEX to the selection of aptamers against a wide variety of targets of interest for biomedical applications, including intracellular targets, could be of great interest.

3.7 References

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Aptamer characterization and affinity determination

4.1 Aptamer characterization

As widely described in literature aptamer-target affinity interaction is affected by pH, ionic strength and the temperature of the binding medium.[1] Many different analytical techniques have been used to characterize aptamer-target binding affinity, such as Isothermal Titration Calorimetry (ITC), Capillary Electrophoresis (CE), Circular Dichroism, Microscale Thermophoresis (MST), Quartz Crystal Microbalance (QCM), Photonic Crystal Surface Wave (PC SW), Surface Plasmon Resonance (SPR) and electrochemical detection.[2,3] Capillary Electrophoresis and Circular Dichroism are able to detect conformational changes in the aptamer structure when bonded to the target, while QCM, PC SW, MST and SPR are able to give real-time response. With other techniques such as electrochemical detection it is usually necessary to label the target or the aptamer.

Several factors must be considered when the aptamer affinity for its target is evaluated, such as the sequence length, its composition and the aptamer secondary structure. All of them are crucial elements and any change in these factors can increase or make collapse affinity.[4]

Secondary structure of aptamers may present stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplexes, and these structures can be involved in the binding process. The binding between aptamer-target takes place through hydrogen bond formation and van der Waals, hydrophobic and molecular shape complimentary interactions.[5]

The outcome of affinity between an aptamer and a target interaction is evaluated through dissociation constants, K_d, expressed as shown in Eq. 4.1, where concentrations of all species are expressed in mol/L.

$$K_d = \frac{[Aptamer]\,[Target]}{[Aptamer-Target]} \qquad \text{Eq. 4.1}$$

As the dissociation constant lowers, the affinity of the interaction between aptamer and target increases.

Eq. 4.1 takes the general form of a rectangular hyperbola. K_d can be estimated directly from this binding curve using a nonlinear regression analysis. Although many equations have been introduced in various techniques, all are essentially variant forms of this equation.[6,7] To characterize the binding specificity of selected aptamers: Gli1, Gli4, Gli2D and Gli3D we have used an electrochemical magneto-assay. Moreover, to compare conformational changes, we characterized Gli4 and Gli1 aptamer solutions in aqueous buffer and ethaline using circular dichroism spectroscopy. To identify

the protein fraction interacting with aptamers we performed a variation of the classic Western Blot technique.

4.2 Aptamer affinity determination with an electrochemical magneto-assay

To evaluate the affinity of each aptamer against both the immunotoxic peptide and the whole protein, we employed a magneto-assay with electrochemical detection.
For ease of use and separation, we used magnetic particles on which the peptide or the protein was immobilized. Then, increasing quantities of aptamer were added to a constant quantity of particles. In such a way, it was possible to construct the corresponding binding curves by quantifying the bound aptamer.

4.2.1 Experimental

4.2.1.1 Modification of streptavidin-magnetic particles with 33-mer

50 µL of strep-MPs were washed twice with 1 mL PBS + 0.01% Tween-20 and resuspended in PBS containing 2 µM of biotinylated 33-mer. Streptavidin-biotin interaction was conducted for 30 min at 30 °C under continuous shaking in a Thermomixer. Afterward, the magnetic beads were washed with PBS + 0.01% Tween-20 and blocked with 500 µM biotin in PBS + 0.01% Tween-20 for 30 min. After two washes, magnetic particles were reconstituted in 500 µL of ethaline.

4.2.1.2 Modification of tosylactivated-magnetic particles with PWG

165 µL of Dynabeads® M-280 Tosylactivated magnetic beads were washed with 1 mL of 0.1 M phosphate buffer (pH 7.4) and then coated with 0.66 mg/mL of PWG under continuous shaking at 37 °C for 18 h. Then, the supernatant was removed and a 0.5% solution of BSA, prepared in PBS, was added and let under continuous shaking at 37 °C for 1 h. After two washing steps with PBS + 0.1% of BSA the beads were resuspended in 250 µL of ethaline.

4.2.1.3 Binding assays

10 µL of the 33-mer modified strep-MPs or of the tosylactivated-MPs with PWG were equilibrated with 490 µL of increasing concentrations of each biotinylated aptamer in ethaline for 1 h at 40 °C. After that, beads were subjected to two washing steps with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20, and then incubated with 500 µL of 2.5 µg/mL strep-HRP conjugate for 30 min under shaking at 30 °C. After two washing steps with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 and one with only 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$, the MPs were resuspended in 10 µL.

4.2.1.4 Temperature and incubation-time optimization

Before constructing all affinity curves, some parameters have been optimized. Considering that deep eutectic solvents are highly viscous liquids[8] and that their viscosity decreases with temperature, as shown in Table 4.1 for ethaline, we carried out the interaction step between magnetic particles modified with the target and the aptamer at different temperatures. The interaction step was carried out completely in ethaline.

ChCl-DES	Viscosity (25°C) mPa s	Viscosity (55°C) mPa s
Ethaline	37	24

Table 4.1 Ethaline viscosity values at different temperatures.

As shown in Figure 4.1, the signal obtained increases with as the temperature. This is explained by a lowering of viscosity which causes a facilitation of the aptamer-target interaction. Moreover, another important parameter to obtain good signal is the incubation time. For these reasons, the test was carried out employing Gli1 at a concentration of 500 nM at two different temperatures, 30 °C and 40 °C, and between 15 and 75 min.

Considering the results obtained the best compromise to develop an assay were 40 °C and 60 min of incubation, (see Figure 4.1).

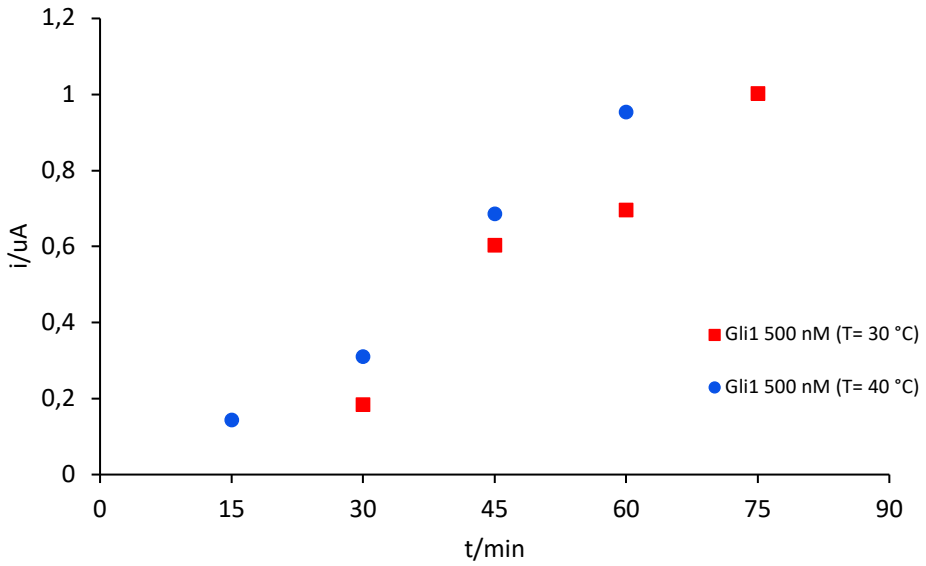


Figure 4.1 Optimization of temperature and time for the assay.

4.2.1.5 Electrochemical quantitation

After enzymatic labeling, the amount of bound aptamer was evaluated electrochemically during the assay. Magnetic beads were collected on the working electrode of a disposable screen-printed carbon electrochemical cell and collected with a magnet (diameter 4 mm) placed under it. After 1 min, 40 µL of TMB solution was added. The current due to the reduction of the product generated during 1 min of enzymatic reaction was recorded at 0 V chronoamperometrically.

In this measurement, the enzymatic reaction produced by HRP is exploited. Horse radish peroxidase, in the presence of TMB and hydrogen peroxide, is able to generate the oxidized form of the TMB, which can be quantified both electrochemically and optically. In this case, the current was measured by applying a constant potential of 0 V, which allows the reduction of the enzymatically oxidized TMB (see Figure 4.2). We considered the average current value of the last 10 seconds of the measurement. The overall assay is sketched in Figure 4.3.

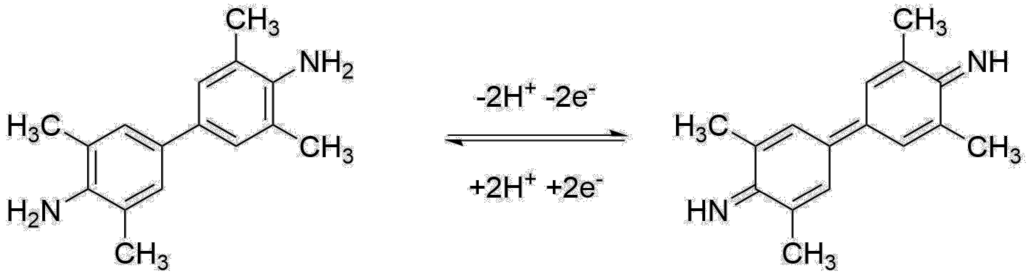


Figure 4.2 Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine

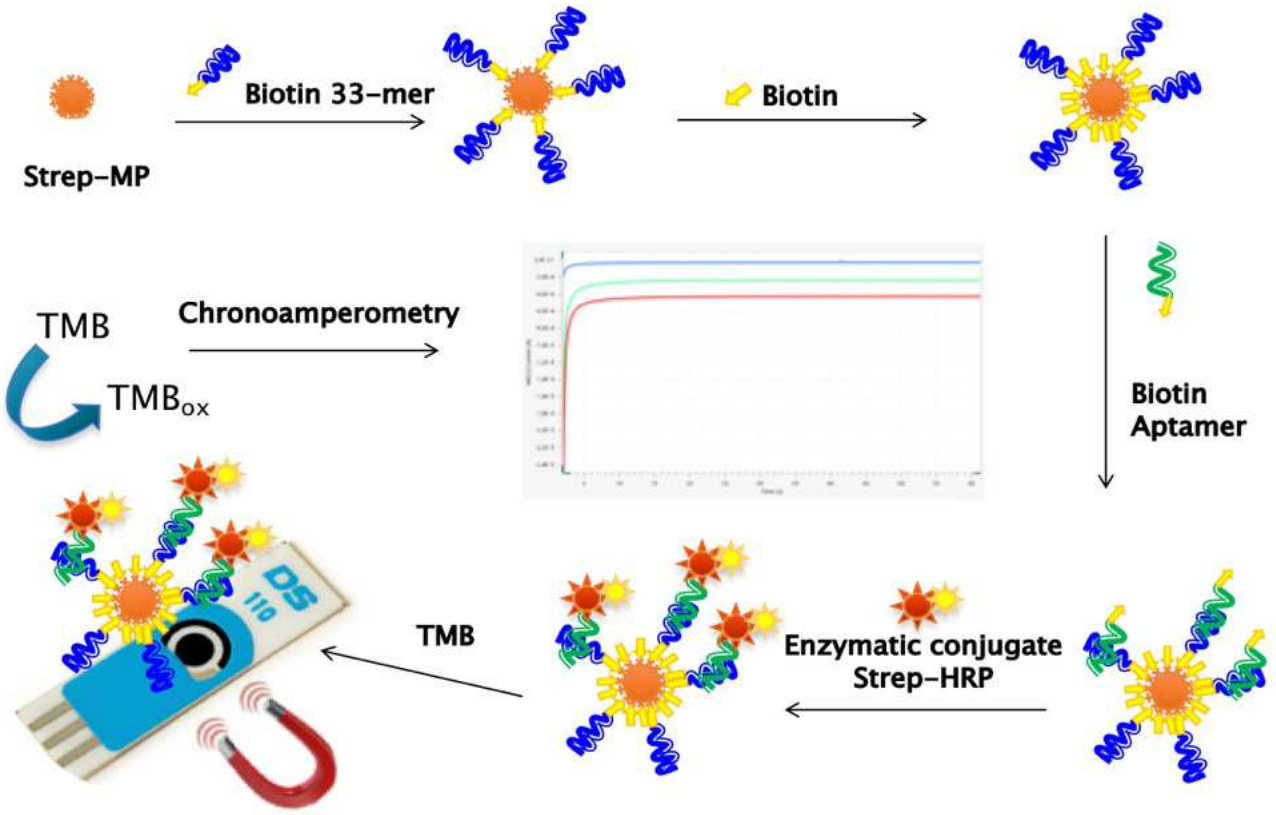


Figure 4.3 Scheme of the assay for obtaining the binding curves.

4.2.1.6 Magnetic particle quantity optimization

The amount of magnetic particles deposited on the working electrode was also optimized. In order to obtain the greatest possible signal, the electrochemical quantification was performed with different quantities of magnetic particles between 1 and 10 µL. As reported in Figure 4.4, the highest signal was obtained with the largest amount of particles. Accordingly, we carried out all measurements with 10 µL of modified magnetic particles.

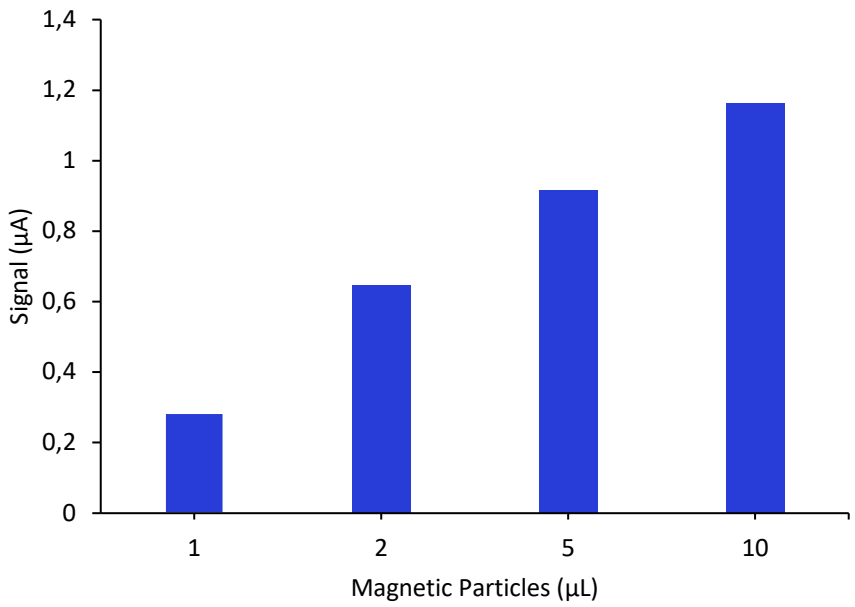
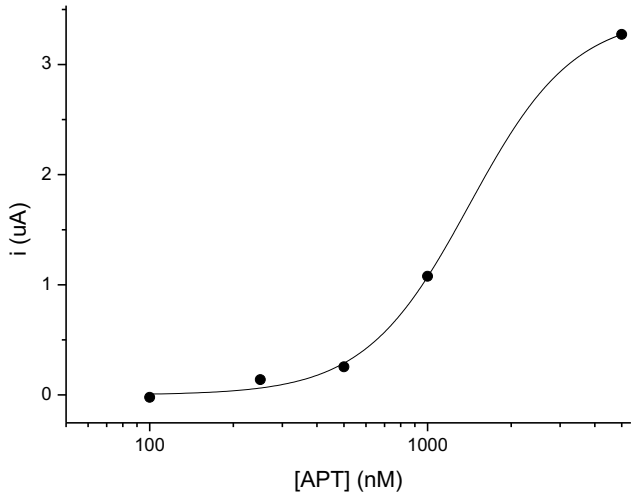


Figure 4.4 Optimization of magnetic particles quantity.

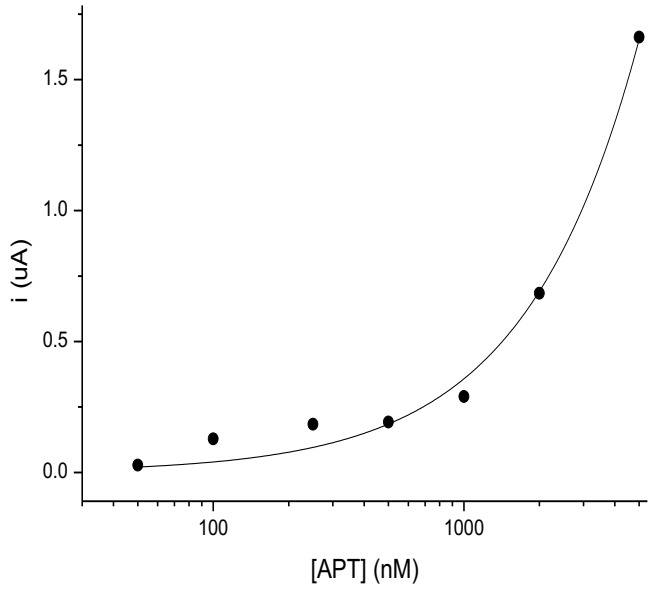
4.2.1.7 Binding curves

After performing the assay, we obtained the binding curves for each aptamer (see Figure 4.5). The observed current increased with increasing aptamer concentration when all aptamers were challenged to the immobilized 33-mer and the native protein. The binding curves were fitted to the Hill equation, giving the dissociation constants reported in Table 4.2.

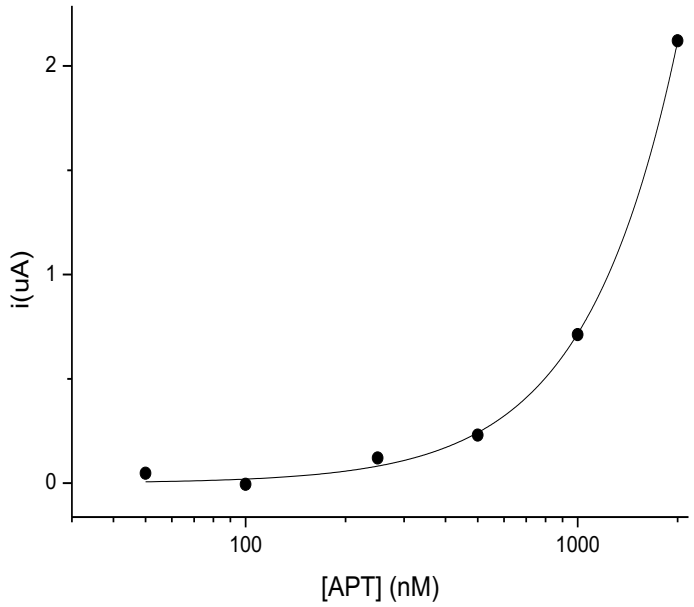
a) Gli1



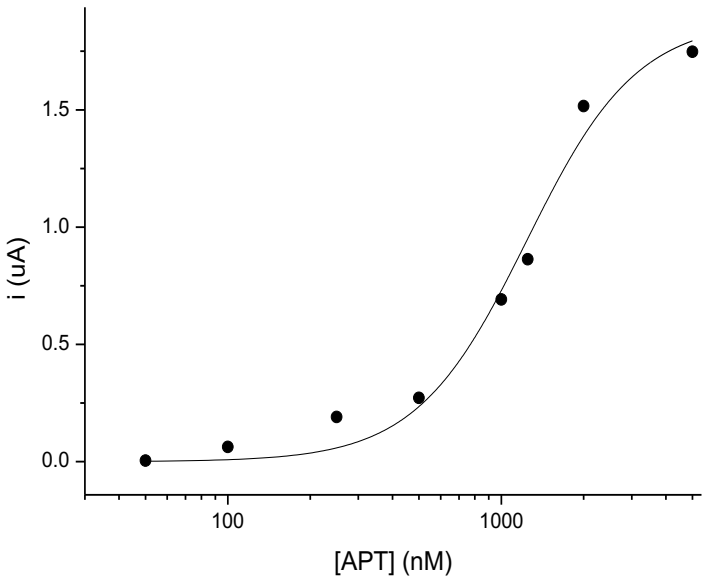
b) Gli4



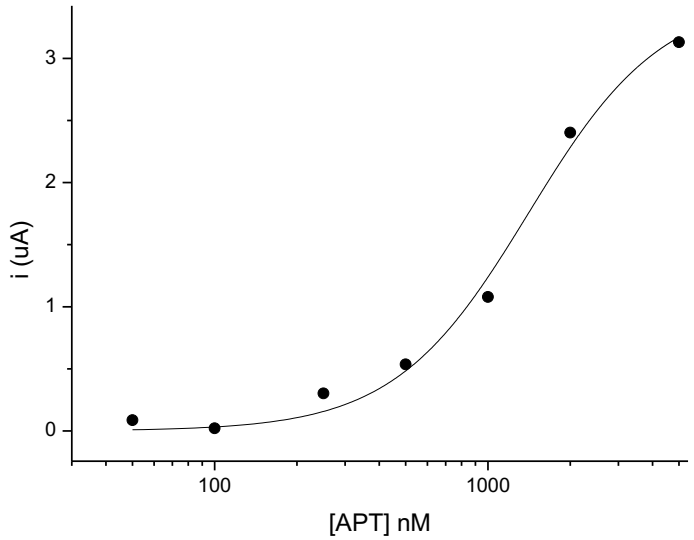
c) Gli2D



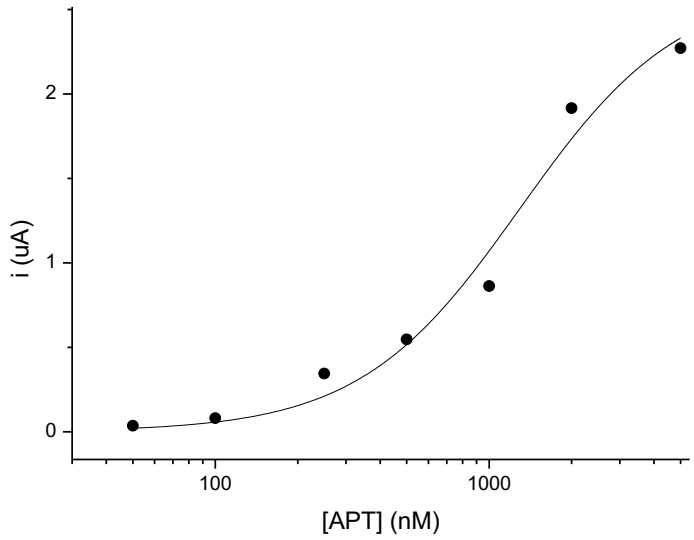
d) Gli3D



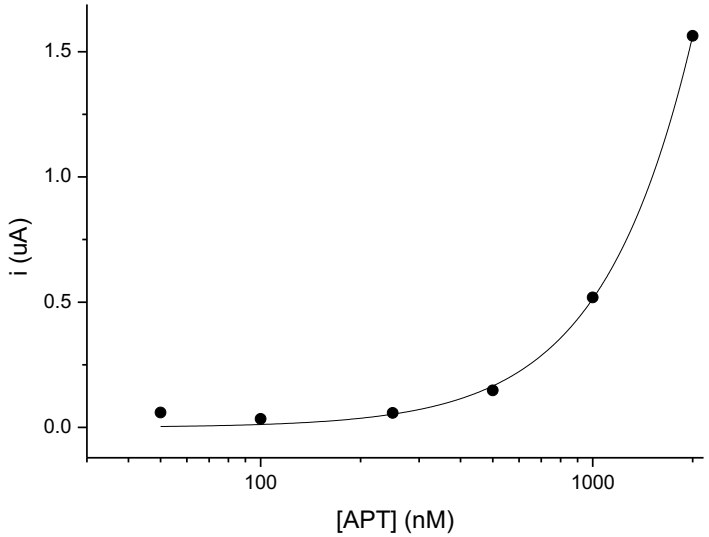
e) Gli1



f) Gli2



g) Gli2D



h) Gli3D

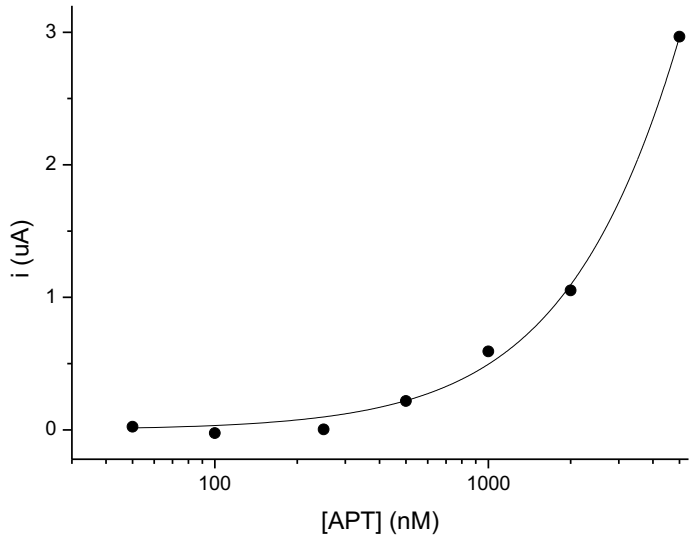


Figure 4.5 Aptamers binding curves against 33-mer (a, b, c, d) and PWG (e, f, g, h).

	Gli1	Gli4	Gli3D	Gli2D
33-mer	1.4 ± 0.1	*	1.3 ± 0.1	*
PWG	1.4 ± 0.2	1.3 ± 0.4	25*	13*
*Poor fitting to the Hill equation				

Table 4.2 Dissociation constants (μM) of complexes of the aptamers with 33-mer peptide and protein.

4.2.2 Discussion

Gli1 and Gli4 retained their activity to recognize both the peptide and the whole protein in ethaline (see Figure 4.6). In contrast to aqueous media, where Gli4 exhibited the highest affinity and the same behavior for both targets,[9] in DES only Gli1 was able to bind with the same affinity to PWG and the 33-mer peptide. This fact highlights the importance of using DES as selection media, where both aptamer and target can adopt conformations different from those shown in water. The most abundant sequence, Gli1, is also the best binder in ethaline, regardless the peptide used as target is included within the natural protein. Surprisingly, Gli4, which was the best binder in aqueous media, displayed in ethaline similar affinity to Gli1 towards PWG, although with ~75% of signal at saturation, but poor affinity to 33-mer peptide. Saturation level was not obtained for the 33-mer peptide-Gli4 system at concentrations of aptamer in the range of our assay (up to 5 μM), and consequently we were unable to estimate the affinity constant. These results may be indicative of a change in the secondary structure of DNA in ethaline, in agreement with our hypothesis that DES might favor a structural selection of folded states that cannot be accessed in aqueous solutions. Aptamer Gli3D displayed the opposite behavior, with the same affinity as the best binder towards the peptide but ~10 fold reduced affinity to the whole protein.

The other aptamer, Gli2D, including only the first motif, displayed poor affinity towards both peptide and protein. This means that the presence of one of the motifs (see Table 3.5) allows the recognition of the peptide within the native protein, but the affinity is of an order of magnitude superior when both are present. Note that a variation of the second motif also appears in Gli4. Recognition of 33-mer with high affinity requires the second motif.

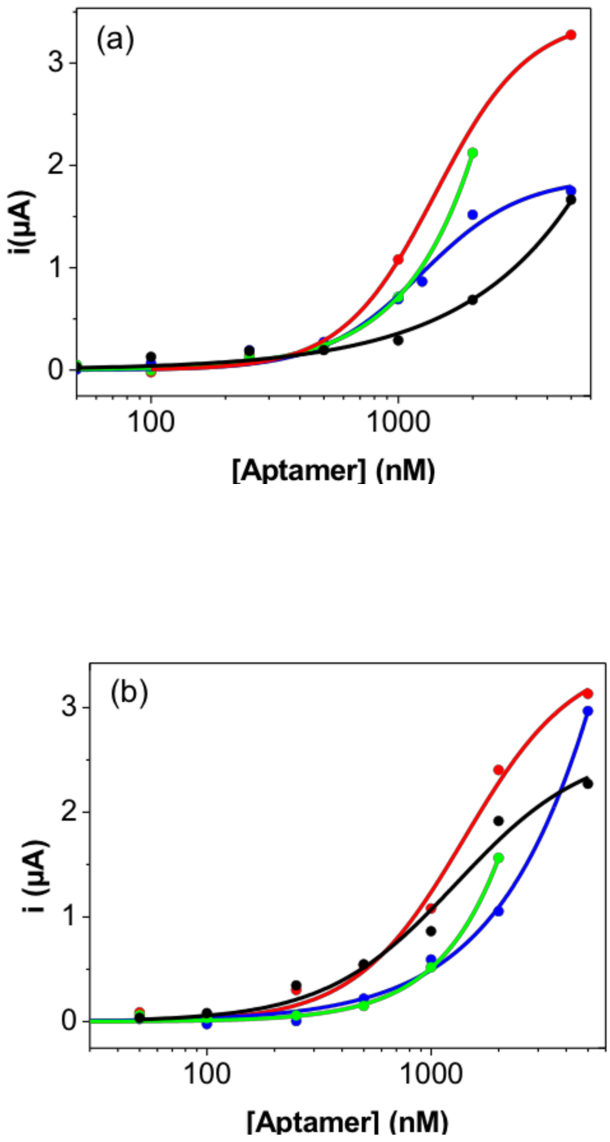


Figure 4.6 Resume of binding curves, (a) against 33-mer, (b) against PWG-Gliadin, in red Gli1, in black Gli4, in green Gli2D and in blue Gli3D.

4.3 Circular Dichroism

Circular dichroism is based on the differential absorption of left and right-handed circularly polarized light, which is a spectroscopic property uniquely sensitive to the conformation of molecules, and so has been very widely used in the study of biomolecules. This technique often provides important information about the function and conformation of biomolecules that is not directly available from more conventional spectroscopic techniques, such as fluorescence and absorbance.[10] Moreover, it is significantly sensitive to the conformational states of nucleic acids and it is a powerful tool for the study of the secondary structures and conformations adopted not only by nucleic acids but also by proteins.[11]

In the case of nucleic acids, circular dichroism measures the asymmetry in nucleic acid systems.[12] Circular dichroism effect arises from the asymmetric backbone sugars and by the helical structures often adopted by nucleic acids. Circular dichroism of nucleic acids is commonly used in an empirical manner to provide a signature for a given secondary structure.[13] In addition, this approach is particularly powerful for monitoring structural changes resulting from changes in environmental conditions such as temperature, ionic strength, and pH.

4.3.1 Experimental

We decided to study Gli1 and Gli4 conformational changes under two different conditions, i.e. in aqueous buffer and in ethaline.

Circular dichroism spectra have been obtained on a JASCO J-600 spectropolarimeter with 5 μM oligonucleotide solutions in ethaline and aqueous buffer (50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$). Spectra were recorded in a 0.1 cm quartz cuvette at room temperature, starting from 500 nm to 200 nm, with a resolution of 1 nm, at a scanning rate of 200 nm/min. Each spectrum was smoothed, and the baseline subtracted (see Figure 4.7).

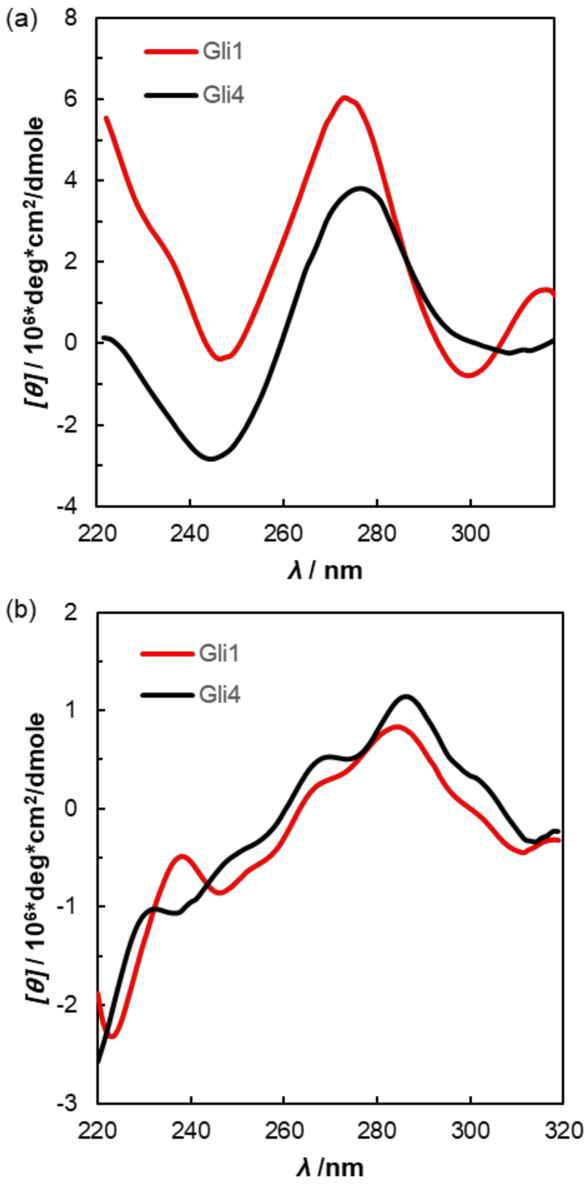


Figure 4.7 Circular dichroism spectra of the selected aptamers, Gli1 and Gli4, in (a) aqueous buffer (50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$) and (b) ethaline solution. Aptamer concentration 5 µM.

4.3.2 Discussion

Samples in aqueous buffer corresponded to DNA conformations which circular dichroism spectra only show maxima around 275 nm and minima around 245 nm, thus suggesting B-helix hairpins, in agreement to the secondary structure of the aptamers predicted by using Mfold. The circular dichroism of the oligonucleotides in ethaline (Figure 4.7b) changes dramatically, with a characteristic negative band at short wavelengths (~220-225 nm) consistent with a change to a conformation of triple helices.

The negative peak, indicative of a Hoogsteen base-pair interaction, is accompanied by a band around 280 nm characterizing the presence of the Watson-Crick intramolecular duplex. The triplex formation, which is a water releasing reaction, may be favored by the dehydration conditions imposed by DES. Additional features of spectra in ethaline may be due to the presence of additional structures.

4.4 Western blot

Western blot analysis, also known as protein immunoblot, is an analytical technique used to detect and quantify specific proteins in a given sample.[14] It was also used as a method for studying antibody specificity and antigen structure. Usually western blots procedures involve SDS–PAGE to separate proteins according to their size, then their transfer to a membrane that can be PVDF or nitrocellulose. Finally, the specific antibody for the target is incubated (primary antibody) and then another antibody (secondary antibody), developed against the first one, is incubated for the detection.[15]

Literature have already reported some modification of this technique employing aptamers. For instance an alternative to Western blot analysis using RNA aptamer-functionalized quantum dots has been reported.[16] However, our idea of changing the primary and secondary antibody with an aptamer labeled with biotin-Strp-HRP was not already described.

4.4.1 Experimental

4.4.1.1 Western-blot protocol

PWG-Gliadin was separated by 10% SDS/PAGE gel for 1.5 h at 50 mA, (see Figure 4.8a) After run, proteins were transferred to a nitrocellulose membrane applying 400 mA ,according to standard western blotting protocols.[17] The membrane was blocked with BSA. Then, biotinylated Gli1, at a concentration of 500 nM in PBS 1X, was incubated for 1 h. After this step, HRP (2,5 ug/mL 1:50) was incubated for half an hour. Then we exploited the horseradish peroxidase (HRP) chemiluminescent reaction based on the catalyzed oxidation of luminol by peroxide. Oxidized luminol emits light when it decays to its ground state. This technique has the speed and safety of chromogenic detection methods, at higher sensitivity levels. In order to perform chemiluminescent detection, we exposed the blot to a suitable X-ray film for an appropriate duration.

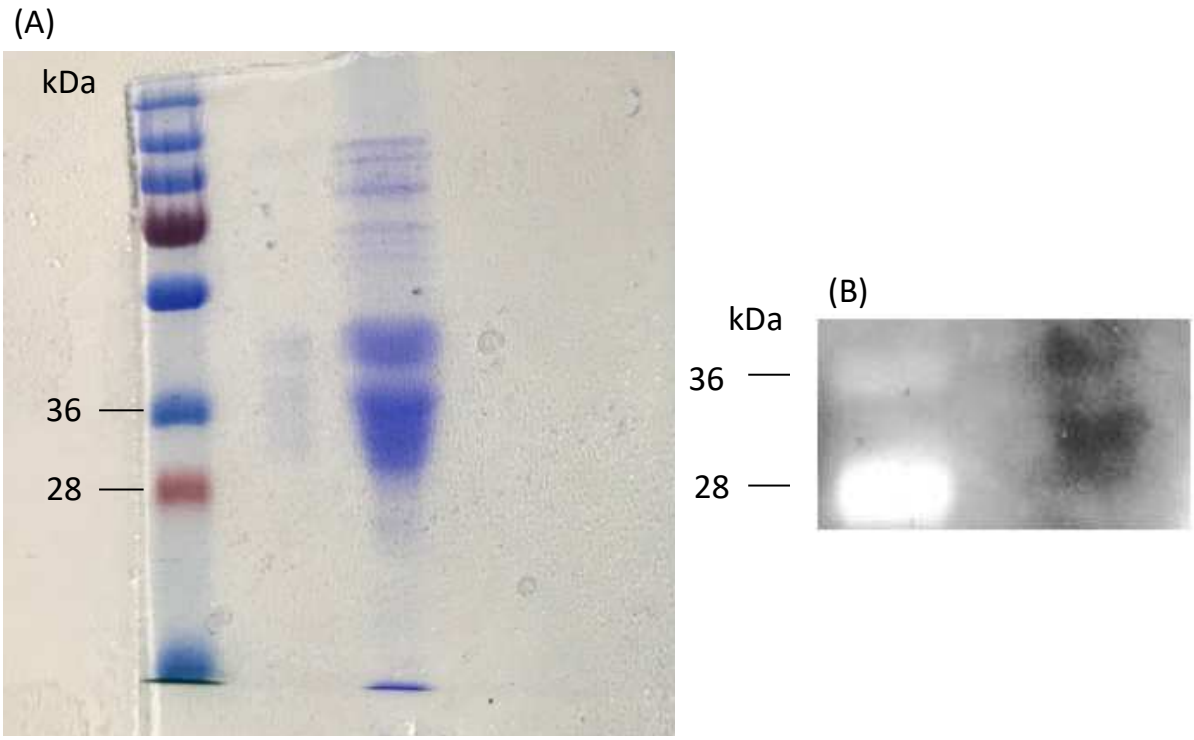


Figure 4.8 (A) SDS-PAGE of PWG Gliadin, (B) Western Blot showing the presence of interaction bands between Gliadin proteins and Gli1 aptamer (500 nM).

4.4.2 Discussion

In this experiment, we used a classical technique with some modifications, to study the interaction of one of the aptamers selected against the PWG-Gliadin, which is actually a large set of different proteins. From the result shown in Figure 4.8, it was possible to identify two bands: one around 30 kDa and the other around 40 kDa, corresponding to α, β and γ gliadin.[18]

Because of the high sensitivity of the Immobilon Western HRP Substrate, we obtained a darker background as shown in Figure 4.8b, but it was still possible to see the bands of interest. The experiment was also performed with an unspecific aptamer, that showed no interaction with our target.

4.5 Conclusions

In summary, binding curves for the selected aptamers were carried out. From the results obtained we can say that Gli1 has the best performance against both peptide and whole protein. Moreover, we studied the conformational changes for both Gli1 and Gli4 in aqueous buffer and ethaline. We have found significant structural differences in the two different media. This can be a useful information for future studies and investigations on DNA's behavior in deep eutectic solvents. Finally, we have successfully obtained a western blot with the use of aptamers, from which we were able to identify exactly the protein fraction with which the interaction takes place. This is a valuable information for future studies and comparisons with the gluten quantification methods currently on the market.

4.6 References

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Gluten quantification assays

5.1 Introduction

As previously mentioned, sandwich ELISA test is currently the official method to quantify gluten[1] using the monoclonal antibody R5 officially recognized by the Codex Alimentarius.[2] However, in commercially available kits also other antibodies are employed such as Skerritt antibody,[3] and G12.[4]

One of the main limitations of these sandwich ELISA tests is that at least two epitopes have to be present simultaneously to be recognized by the monoclonal antibody.[5] For this reason, it is of considerable importance to develop assays using only one recognition element. Consequently, label-free approaches and competitive assays are the best options. In this chapter are presented two different assay approaches employing only one aptamer to overcome this problem.

5.2 First approach: Label-free assay

The possibility of developing a label-free assay was first investigated by conducting experiments based on the use of multiwalled carbon nanotube-modified screen-printed electrodes (MWCNT–SPEs). Aptamers were immobilized on the electrode surface exploiting the presence of carboxylic groups and the effect of steric hindrance resulting from the successive interaction with gliadin was measured electrochemically. Carboxylic groups were naturally present on nanotubes of MWCNT–SPEs or introduced through the electro-polymerization of a substrate. Aptamers were modified in the 5' position with an amino group to allow the immobilization on the electrode surface through amide bond formation. Differential pulse voltammetry (DPV) has been used in order to characterize each modification step of the SPE biosensor surface. With this purpose, the decrease in current of the ferricyanide reduction peak during the interaction between aptamer and analyte was monitored. In Figure 5.1 is reported a scheme of the biosensor assembly.

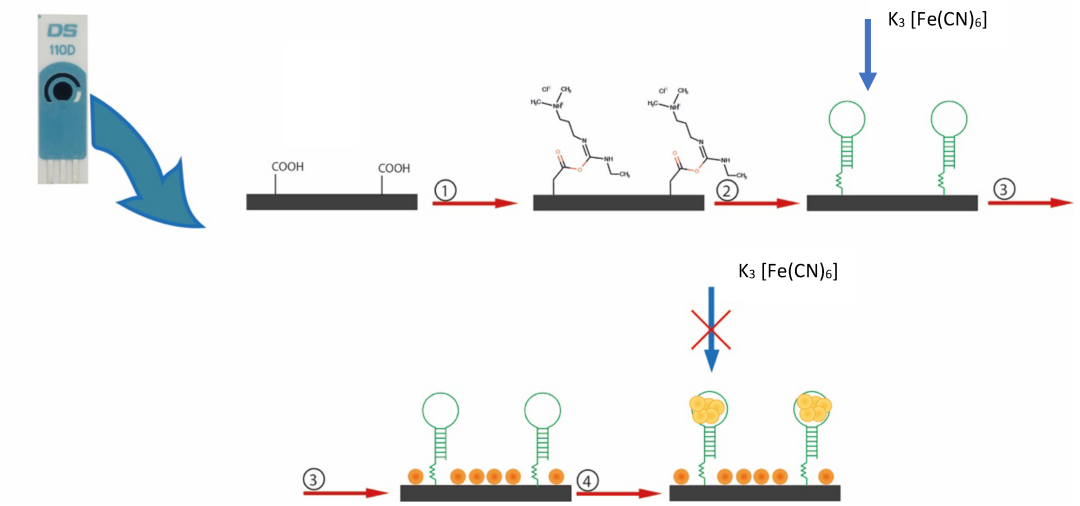

Figure 5.1 Schematic representation of the sensor (1 EDC/NHS, 2 Aptamer, 3 BSA, 4 Gliadin).

For electrochemical measurements, DPV was used. This technique is among the most sensitive electroanalytical techniques for the direct concentration detection, that finds wide use for trace analysis. In differential-pulse voltammetry, fixed-magnitude pulses superimposed on a linear potential ramp are applied to the working electrode (see Figure 5.2a). The current is sampled twice, just before the pulse application and at in the pulse end.[6] In this way this differential reading of the current generates a peak shaped voltammogram (see Figure 5.2b).

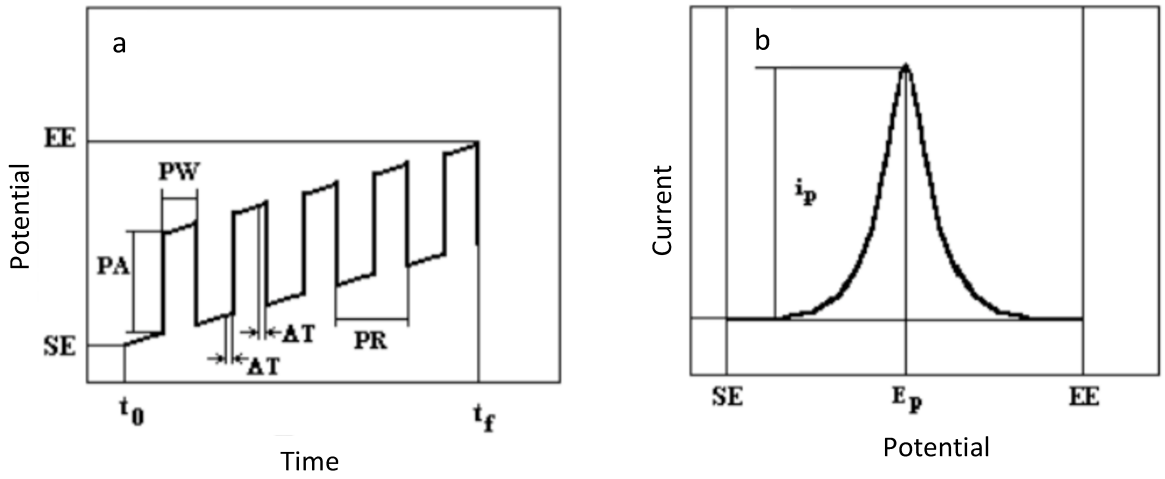

Figure 5.2 Differential Pulse Voltammetry (a, anodic potential scanning; b, plot of voltammogram).

5.2.1 Experimental

5.2.1.1 Reagents and apparatus

All chemicals used in the study were of analytical reagent grade purity. Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), hexammineruthenium (III) chloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), Bovine Serum Albumin (BSA) and salts for buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA). o-aminobenzoic acid (o-ABA) was obtained from Fluka. Ultrapure water purified by an Elgastat UHQ-PS system (Elga Lab. Water, Siershahn, Germany) was employed to prepare buffers and solutions. MWCNT–SPEs were purchased by Dropsens (Metrohm Italiana srl). Voltammetric measurements were performed with an Autolab PGSTAT 30 potentiostat (Ecochemie, Utrecht, NL) driven by the relevant GPES software installed on a Pentium X computer.

5.2.1.2 Electropolymerization of o-aminobenzoic acid (o-ABA)

Poly o-aminobenzoic acid (PABA), is a carboxyl functionalized aniline conductive polymer. Its carboxylic acid group serves as a functional group that can be used to immobilize aptamers or other molecules of interest through covalent bonds.

The electropolymerization of o-aminobenzoic acid (o-ABA) at MWCNT–SPEs was achieved by applying potential voltammetric cycles from 0 to 1.0 V, at a sweep rate of 50 mV/s in 1 M H_2SO_4, 0.1 M KCl solution containing 50 mM o-aminobenzoic acid for 15 cycles (see Figure 5.3).[7] After polymerization, the electrode was rinsed with 1 M H_2SO_4 and Milli-Q water to remove the monomer excess.

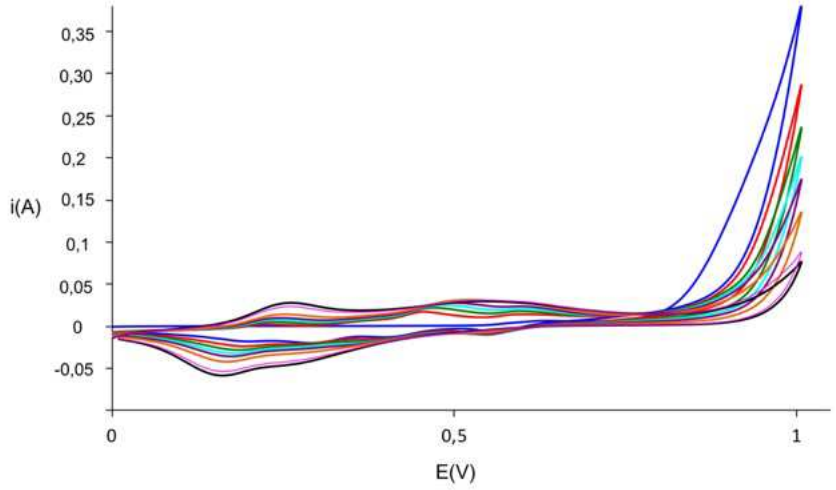

Figure 5.3 Cyclic voltammograms of o-ABA polymerization at SPE.

It is assumed that the o-ABA was coupled by a head-to-tail for growing polymeric chains in the main path (see Figure 5.4). This procedure enabled a surface rich in carboxylic acids to be obtained, which was ready to be subsequently modified.

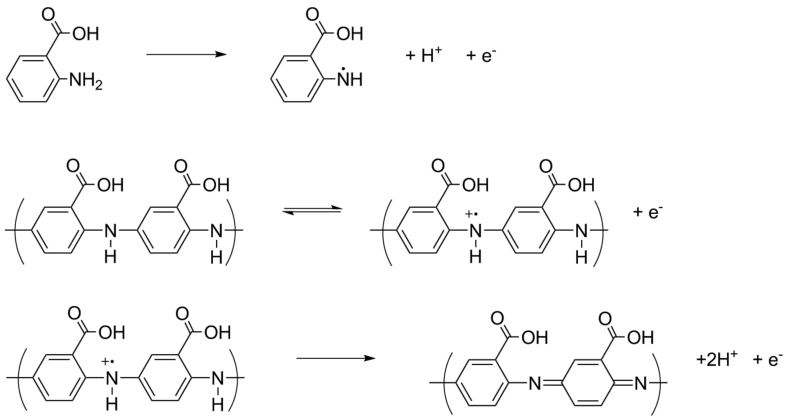

Figure 5.4 o-ABA polymerization.

5.2.1.3 Activation of carboxyl groups

The most effective and versatile method to achieve conjugation to carboxylic acids (–COOH) is that of using carbodiimide compounds.[8] The most readily available and commonly used carbodiimide in water is EDC for aqueous crosslinking. Carbodiimide conjugation, takes place by activating carboxyl groups in a direct reaction with primary amines via amide bond formation. Because no portion of their chemical structure becomes part of the final bond between conjugated molecules, carbodiimides are considered zero-length carboxyl-to-amine crosslinkers. Moreover, N-hydroxysuccinimide (NHS) or its analog (Sulfo-NHS) is often included in EDC coupling protocols to improve efficiency or create dry-stable (amine-reactive) intermediates.[9] EDC couples NHS to carboxyls (see Figure 5.5), forming an NHS ester that is considerably more stable than the O-acylisourea intermediate, thus allowing an efficient conjugation to primary amines at physiologic pH.

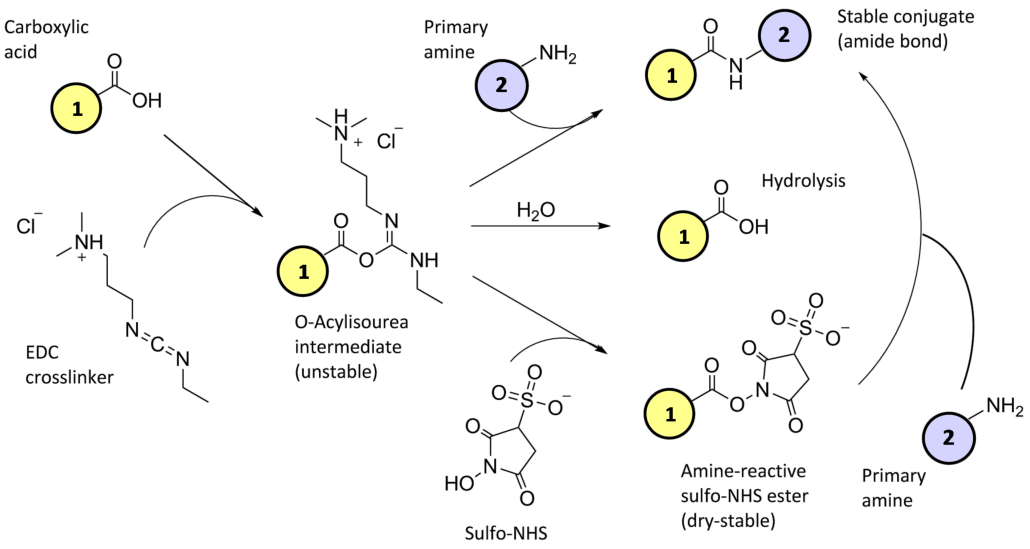

Figure 5.5 Carboxyl-to-amine crosslinking using the carbodiimide EDC and sulfo-NHS.

Hence, the carboxyl groups were activated by 0.4 M EDC and 0.2 M NHS solution prepared in 0.1 M PBS buffer (pH 5.0), in order to immobilize the 5' amino-modified aptamer. The NHS/EDC mixture was added for 1 h.

5.2.1.4 Aptamer incubation and surface blocking

After surface activation, the aptamer at a concentration of 1 μM was incubated for 1 h. Then, after washing, the electrode was incubated with BSA (1%) for 30 min to prevent non-specific interactions. Each step of the electrode surface modification was followed by differential pulse voltammetry.

5.2.1.5 Evaluation of aptamer surface coverage by the Tarlov method

With the Tarlov method it is possible to quantify the surface density of DNA immobilized on an electrode surface.[10] In this method a DNA-modified electrode is placed in a low ionic strength electrolyte containing a cationic redox marker. Redox cations exchange for native counter-ions associated with the nucleotide phosphate residues of the probe. The quantity of redox marker "electrostatically trapped" at the DNA-modified electrode is then determined using chronocoulometry.

The DNA coverage of the electrode surface was calculated from the number of cationic redox molecules electrostatically bound to the anionic DNA backbone. The association constant between cations and DNA phosphate increases with the cation charge. When an electrode modified with DNA

is placed in a low ionic strength electrolyte containing a multivalent redox cation, the redox cation exchanges with the native charge compensation cation (potassium) and becomes electrostatically trapped at the interface. Then, the quantity of cationic redox marker can be measured using chronocoulometry, a current integration technique, under equilibrium conditions.

An interesting aspect of chronocoulometry is that the charge due to species adsorbed onto the electrode surface can be differentiated from the charge due to redox molecules freely diffusing to the electrode surface. Therefore, measurements of surface-confined redox species can be made in the presence of solution of the redox marker. The integrated current, or charge *Q*, as a function of time *t* in a chronocoulometric experiment is given by the integrated Cottrell expression (Eq. 5.1).

$$Q = \frac{2nFAD_0{}^{1/2}C_0{}^*}{\pi^{1/2}} t^{1/2} + Q_{dl} + nFA\Gamma_0 \qquad \text{Eq. 5.1}$$

where *n* is the number of electrons involved in the reduction of each molecule, *F* is the Faraday constant (C/equiv), *A* the electrode area (cm^2), D_O the diffusion coefficient (cm^2/s), C_O* the bulk concentration (mol/ cm^3), Q_{dl} the capacitive charge (C), and $nFA\Gamma_O$ the charge from the reduction of Γ_O (mol/cm^2) of adsorbed redox marker. The term Γ_O represents the amount of redox marker confined near the electrode surface. The chronocoulometric intercept at *t*=0 is then the sum of the double-layer charging and the surface excess terms. The surface excess is determined from the difference in chronocoulometric intercepts obtained by identical potential step experiments performed in the presence or absence of redox marker.

The saturated surface excess of the redox marker is converted to DNA probe surface density with the relationship described in Eq. 5.2.

$$\Gamma_{DNA} = \Gamma_0(z/m)\ (N_A) \qquad \text{Eq. 5.2}$$

Where Γ_{DNA} is the probe surface density in molecules/cm^2, m is the number of bases in the DNA probe, z is the charge of the redox molecule, and N_A is the Avogadro's number.

In our experiments, this method has been applied to determine aptamer surface coverage. With this purpose we measured the charge associated with the reduction of ruthenium (III) to ruthenium (II). The ruthenium solution was deaerated with nitrogen and protected from light. After

coloumetric measurements it was possible to obtain Q of ruthenium and consequently to calculate the DNA molecules per cm^2.

In Table 5.1, it can be seen that the electropolymerized electrode provided the greatest amount of DNA on the electrode surface.

Modifications of MWCNT–SPEs	**DNA molecules/cm^2**
EDC 0.4 M in HEPES (pH=7)	1,76661E+12
EDC 0.4 M + NHS 0.2 M in PBS (pH=5)	4,71095E+12
o-ABA electropolymerized SPE + EDC 0.4 M + NHS 0.2 M in PBS (pH=5)	5,79327E+13

Table 5.1 DNA molecules/cm^2 obtained with Tarlov method on MWCNT–SPEs.

The results obtained pointed out that the electro-polymerization of the o-ABA causes the immobilization of a greater quantity of aptamer with respect to the simple use of the -COOH residues naturally present on MWCNT–SPEs. Hence, we decided to use electro-polymerized electrodes for the next steps of the assay.

5.2.2 Results and discussion

5.2.2.1 Electrochemical label-free assay for gluten quantification

Modified electrodes were incubated with growing concentrations of PWG-Gliadin between 2.5 and 50 ppm. To evaluate the interaction between the aptamer and PWG-Gliadin, the biosensor was exposed to different concentrations of PWG-Gliadin (2.5, 5, 7.5,10 and 15 ppm) and after each incubation, the decrease of the reduction of ferricyanide was evaluated. Figure 5.6 shows the decrease of the reduction peak current recorded by differential pulse voltammetric for ferricyanide with the increase of PWG-Gliadin on MWCNT–SPEs. The inset in Figure 5.6 shows a good linear relationship between these cathodic peak currents and the logarithm of PWG-Gliadin concentrations between 2,5 and 15 ppm.

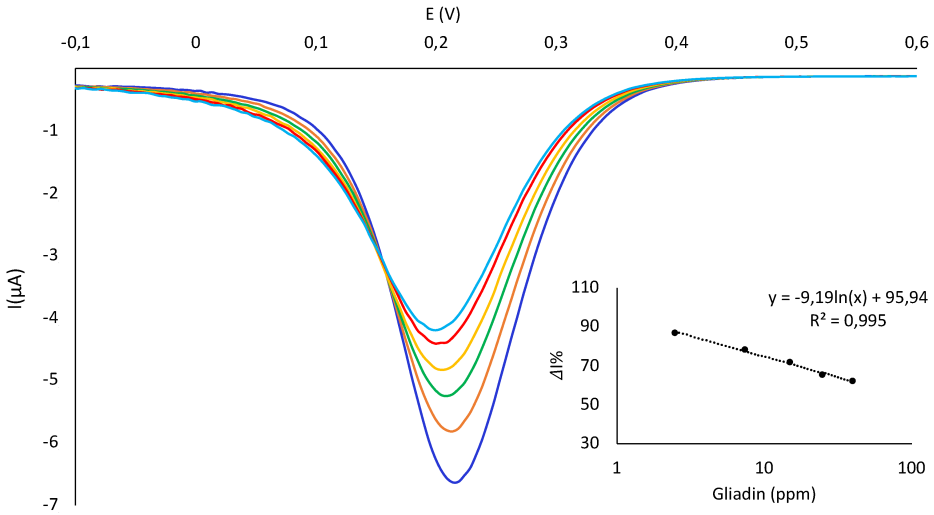

Figure 5.6 Differential pulse voltammetry of ferricyanide with the increase of PWG-Gliadin.

Even though these results showed that this approach could be applied to the determination of gluten in food, its sensitivity was not satisfactory because it does not provide concrete improvements against usual, more effective and simple methods so far adopted. Therefore, we decided to take another path for the development of a more effective assay.

5.3 Second approach: Electrochemical competitive assay on magnetic particles

Starting from a competitive assay reported in literature,[11] we developed a competitive magnetic-particle assay to quantify gluten in which the interaction step between the aptamer and the target, took place directly in ethaline.

This approach allowed gluten to be extracted from food and directly quantified without the need of dilutions, unlike commercial kits. In fact, cocktail solutions for the extraction present in kits available on the market are incompatible with the subsequent assay, so that large dilutions are always necessary. Obviously, this leads to some inaccuracies in the analytical quantification.

5.3.1 Experimental

5.3.1.1 Reagents and Apparatus

5'-tagged (biotin) aptamers were obtained from Laboratorios CONDA (Madrid, Spain). Peptide 33-mer was obtained from Biomedal (Sevilla, Spain). ssDNA sequences employed in the assay are summarized in Table 5.2. Gliadin standard from Prolamin Working Group (PWG) was acquired to R-Biopharm AG (Germany). Gluten from wheat was acquired from Sigma-Aldrich (Spain). Ethaline was supplied by Scionix Ltd. (London, UK) and employed as received. All aqueous solutions were prepared with water purified with a MIlliQ system (Millipore, Spain). Salts for buffer solutions, Tween-20, Bovine Serum Albumin (BSA), 1 M TRIS/HCl pH 7.4, Phospate Buffered Saline (PBS) 10× and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA solution were obtained from Sigma-Aldrich (Spain). Dynabeads™ MyOne™ Streptavidin C1 (strep-MPs), Dynabeads® M-280 Tosylactivated, DNA and Protein Lo-bind Eppendorf tubes, Eppendorf ep T.I.P.S PCR and Streptavidin-peroxidase conjugate (strep-HRP) were obtained from Thermo Fisher Scientific. Screen Printed Electrodes were purchased by Dropsens (Spain). Electrochemical measurements were performed with a computer-controlled µ-AutoLab type II potentiostat with the Nova 2.1 software (EcoChemie, The Netherlands). Thermomixer (Eppendorf Iberica, Spain) and the magnet (DynaMag-2) for magnetic separation were purchased from Life Technologies (Madrid, Spain).

Name	Sequence
Gli1	CTAGGCGAAATATAGCTACAAC TGTCTGAAGGCACCCAAT
Gli4	CCAGTCTCCCGTTTACCGCGCCTACACATGTCTGAATGCC

Table 5.2 Sequences of employed aptamers.

5.3.1.2 Food samples extraction

Extraction of gluten from food was performed using ethaline. About 0.35 g of the powdered sample were extracted in vials with 3.5 mL of pure DES. These vials were shaken by a vortex for 2 min, and then they were left in a water bath at 55 °C for 45 min. After this time, they were shaken again for 2 min and centrifuged for 10 min at 5000 rpm. After extraction, samples were directly subjected to quantification by the competitive assay.

5.3.1.3 Competitive assay procedure

Magnetic particles were functionalized with 33-mer as already described in section 4.2.1.1. Biotinylated aptamer (1 μM) and growing quantities of PWG-Gliadin (1-10.000 ppb) were incubated with fixed amounts of particles modified with 33-mer in ethaline. In this step PWG in solution competed with the peptide immobilized to bind a limited amount of aptamer. As a result, only a part of the biotinylated aptamer remained bound to magnetic particles. Then, the particles were washed twice with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 to separate the aptamer fixed in particles from the rest of the solution. They were subsequently incubated with 500 μL of 2.5 μg/mL strep-HRP conjugate for 30 min under shaking at 30° C. After two washing steps with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 and one with only 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$, MPs were resuspended in 10 μL of buffer. Lastly, the amount of bound aptamer was evaluated electrochemically. Magnetic beads were placed on the working electrode of a disposable screen-printed carbon electrochemical cell and collected with a magnet (diameter 4 mm) placed under itself. After 1 min, 40 μL of TMB solution was added. The current due to the reduction of the product generated within 1 min of enzymatic reaction was chronoamperometrically recorded at 0 V. A schematic representation of the assay is shown in Figure 5.7.

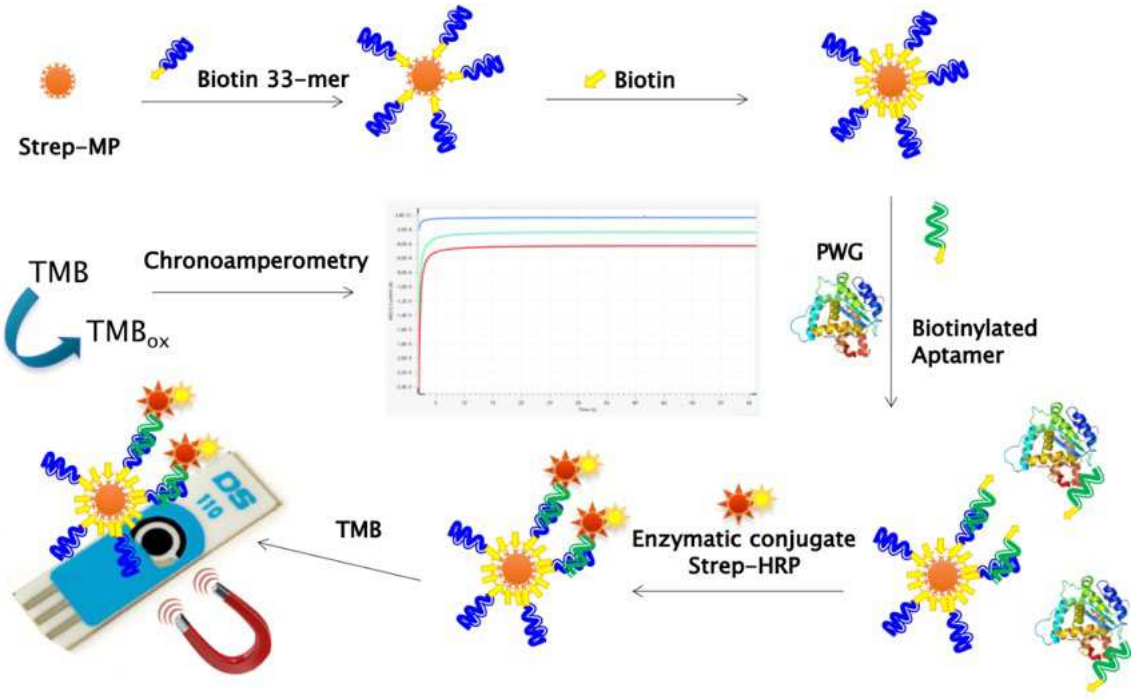

Figure 5.7 Scheme of the competitive assay.

5.3.2 Results and discussion

Figure 5.8 shows the current intensities obtained in the calibration of the competitive assay with Gli 1 using PWG-Gliadin as standard. Data has been adjusted to the four-parameter logistic function:

$$y = A + \frac{(B-A)}{(1+\left(\frac{x}{x_0}\right)^p)} \qquad \text{Eq. 5.3}$$

Where A is the response to an infinite concentration of analyte, B is the response to a zero concentration of analyte, x_0 is the concentration of analyte that provides 50% of the maximum signal and p is the slope of Hill that represents the slope in the turning point of the sigmoid curve.

The competitive assay confirmed the usefulness of the selected aptamer for the direct detection of gluten in ethaline extracts, without extra-dilutions. The presence of gliadin in the ethaline solution effectively reduces the aptamer binding to 64% for a PWG concentration as low as 0.1 ppm, with a further decrease in binding to 50% as PWG increases to 1 ppm, suggesting that the signal change is due to the specific displacement of the bound aptamer by PWG in solution.

The limit of quantification was determined as the lowest concentration point on the calibration curve that this test can reliably detect. The limit of quantification was 1 ppb and the linear working range was found between 10 and 1000 ppb.

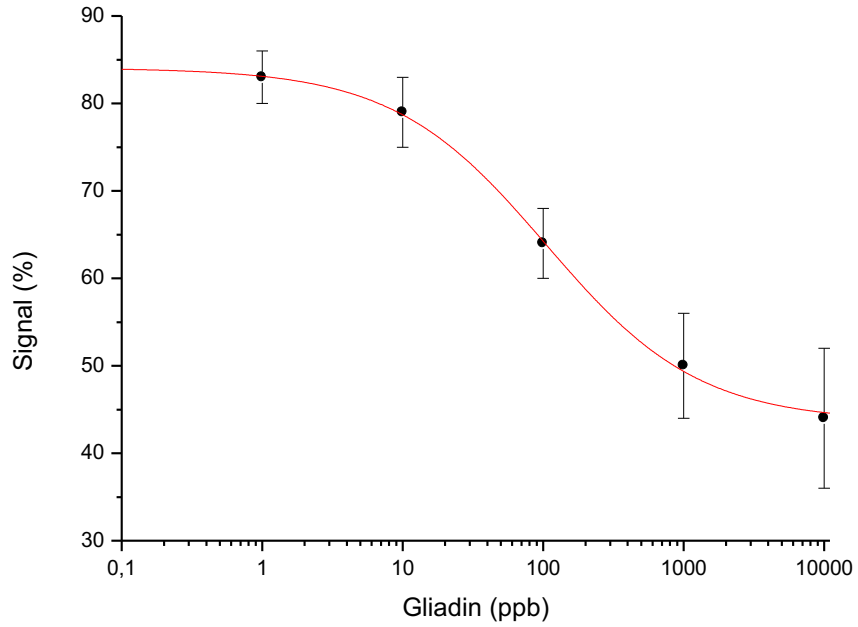

Figure 5.8 Calibration curves for the competitive assay based on the Gli 1 using gliadin PWG as standard.

To verify the applicability of this method, two samples certified by an inter-laboratory trial using the official method based on the R5 antibody were analyzed. These two samples consisted of a cake mix certified as gluten-free in an inter-laboratory trial and an infant soy preparation with a certified value of 21 ppm of gluten also controlled in an inter-laboratory trial. Gluten quantification values for both samples were obtained using the four-parameter logistic function obtained by the calibration. The values obtained were correct by adjusting for the actual weight/volume of sample/extraction solution used in the extraction step and by the actual extract dilution used. The results are summarized in Table 5.3.

Food sample	Signal %	ppm of gluten	Certified value (ppm of gluten)
Cake mix	67±6	1.4	Negative
Infant soy formula	21±3	>20	21

Table 5.3 Results obtained with the competitive assay based on Gli1.

The calibration curve was also constructed using Gli4, but the results obtained were worse than those obtained using Gli1. This can be explained by the fact that Gli4 in ethaline displays different

dissociation constants against the whole protein and the peptide (see Figure 5.9). For this reason, the tests and quantifications have always been made using Gli1.

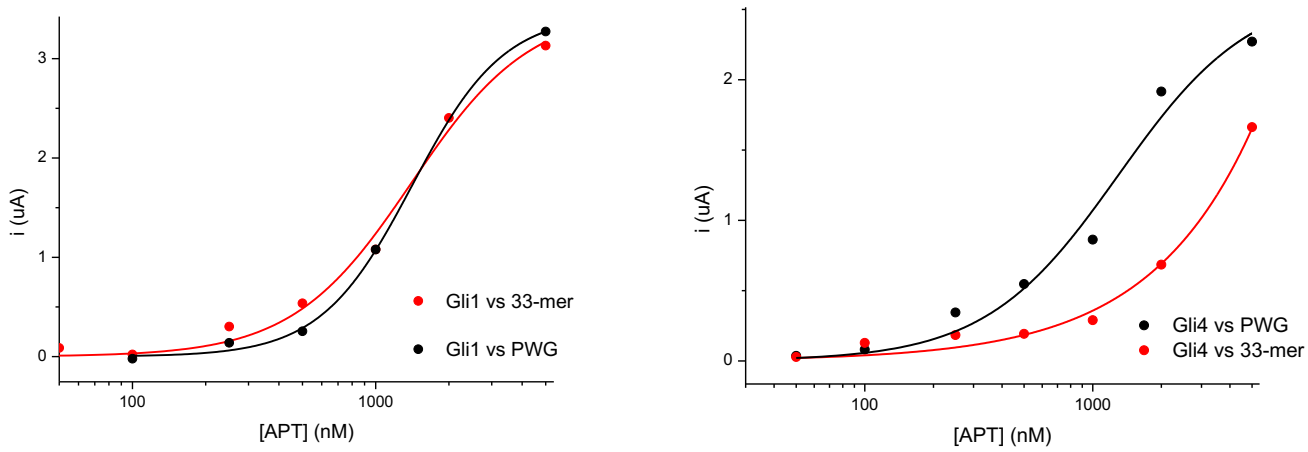

Figure 5.9 Comparison between binding curves of Gli1 and Gli4.

Moreover, the calibration curve was also performed using gluten from wheat in solution as standard. The competitive assay responded positively, giving the curve shown in Figure 5.10 that was also adjusted to the four-parameter logistic function described above. However, by comparing the two curves for quantification purposes, the calibration obtained with the PWG-Gliadin seems to give better results.

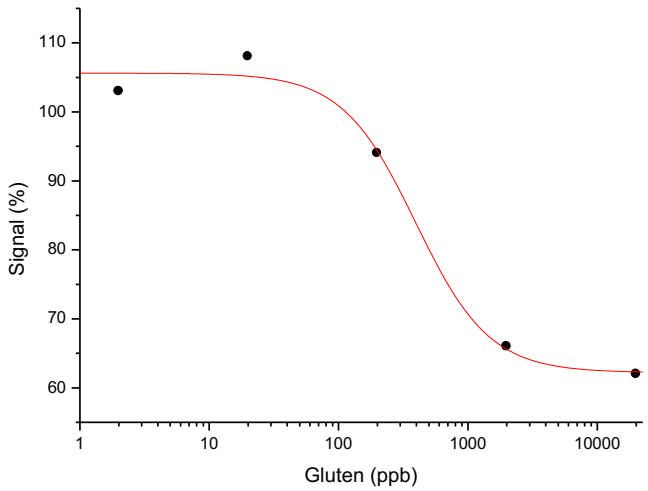

Figure 5.10 Calibration curve for the competitive assay based on the Gli 1 using gluten from wheat as standard.

5.3.2.1 Selectivity

We verified that the method responded only to toxic proteins of gluten and not to other kinds of proteins that may be present in gluten-free food.

To evaluate the cross-reactivity, the response of the assays with both aptamers was measured at increasing concentrations of the proteins extracted from soy and rice flour. Analytical responses, expressed as a percentage of current with respect to the maximum current intensity in the absence of protein, were quite close to 100% as can be seen in Figure 5.11. According to these results, the developed assay does not show cross-reactivity with non-toxic proteins for celiac patients.

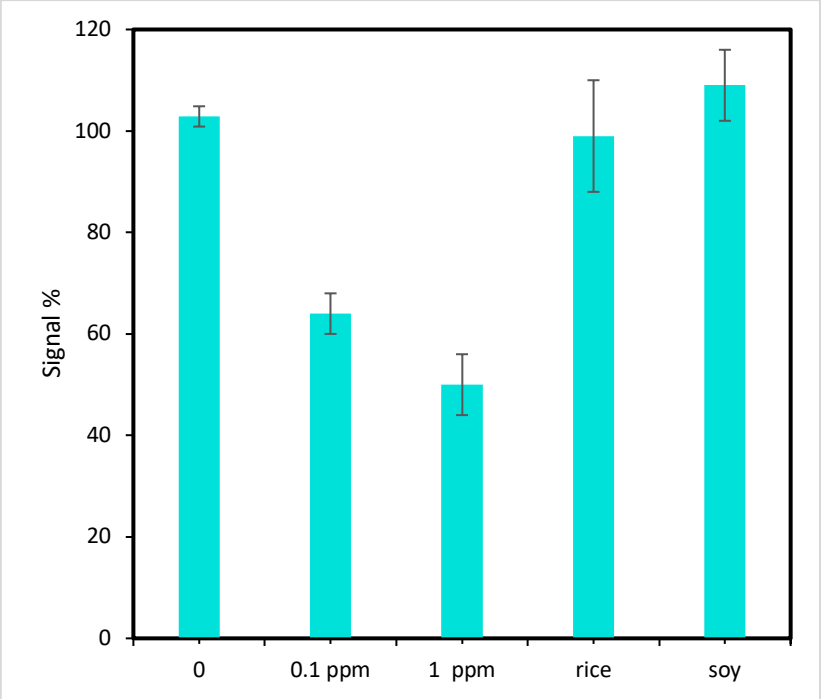

Figure 5.11 Signal change in the competitive assay, using 1 µM Biotin-Gli1 and 0.1 mg/mL of 33-mer modified magnetic beads, for different amounts of standard gliadin and rice or soy flour ethaline extracts.

5.4 Conclusions

The label-free approach did not provide satisfactory results, in fact, its sensitivity compared with the commercial kits has proved to be not enough low.

Therefore, we moved toward a competitive assay which demonstrated to be sufficiently sensitive and selective to be applied to the determination of gluten in foods labeled "gluten-free". In fact, our method has a LoQ of 1 ppb of gliadin and a linear working range between 10 and 1000 ppb, while commercially available kit such as GlutenTox ELISA Competitive (employing G12 antibody) has a LoQ of 1.5 ppm of gliadin and RIDASCREEN Gliadin Competitive (employing R5 antibody) has a LoQ of 5.0 ppm of gliadin.[12] Moreover, the most innovative and important aspect of our approach is the possibility to analyze directly food samples extracted with ethaline without the need for dilutions, providing more accurately quantitation.

5.5 References

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Conclusions and future perspectives

6.1 Conclusions and future perspectives

The main focus of this thesis was the development of a new quantification method for gluten in food. The rationale for pursuing such an objective was that gluten quantification still encounters significant difficulties resulting from the identity of the allergen, the lack of an effective and universal extraction method and the availability of few receptors of sufficient affinity and selectivity suitable for obtaining a reliable analytical method.

Based on these assumptions, we evaluated the possibility of employing deep eutectic solvents (DESs) as extractants combined with the use of aptamers as recognition elements. For this purpose, the suitability to extract gliadin (50% content of gluten) of two DESs, ethaline and reline, was evaluated employing a commercially available ELISA kit. The results obtained pointed out that DESs are very promising as gluten extraction solvents from both unprocessed and processed food. In particular, ethaline provided really good and reliable results. Consequently, we decided to perform the selection of aptamers able to recognize gliadin in ethaline. We used SELEX, which is a universal and iterative process, where an initial degenerated library of oligonucleotides is challenged against the analyte, to select sequences with the highest affinity. First, we took a further step by demonstrating the viability of SELEX in a green extraction solvent, thus providing a method for obtaining aptamers able to recognize non-soluble and poorly water-soluble molecules or species prone to aggregation in aqueous solutions.

After SELEX procedure, we performed Illumina sequencing and exploiting bioinformatic analysis, thus selecting four aptamers to test. We characterized them and determined the affinity constants. Lastly, we developed an electrochemical competitive assay sufficiently sensitive and selective to be applied to the determination of gluten in foods labeled "gluten-free", to assess compliance with the law and provide more information to the consumer. The competitive assay confirmed the usefulness of the selected aptamers, in particular Gli1, for the direct detection of gluten in ethaline extracts, without extra-dilutions. We also verified that the method responded only to toxic proteins of gluten and not to other kinds of proteins that may be present in gluten-free food. According to the results obtained, the developed assay does not show cross-reactivity with non-toxic proteins for celiac patients. The limit of quantification of the assay was 1 ppb and the linear working range was found between 10 and 1000 ppb.

The most innovative and important aspect of the approach here presented is the possibility to directly analyze extracts of food in ethaline without the need for dilutions with high sensitivity and

selectivity. This approach could be extended to other poor water-soluble molecules of interest not only in food safety field but also in biomedicine.

The procedure here developed could be further improved. The intrinsic conductivity of deep eutectic solvents could be exploited to perform the whole quantification assay including the electrochemical detection in ethaline. Moreover, the use of other techniques such as electrochemical impedance spectroscopy (EIS) could be considered.

6.2 List of publications

- Svigelj, R., Bortolomeazzi, R., Dossi, N., Giacomino, A., Bontempelli, G., & Toniolo, R. (2017). An effective gluten extraction method exploiting pure choline chloride-based deep eutectic solvents (ChCl-DESs). Food Analytical Methods, 10(12), 4079-4085.

- Svigelj, R., Dossi, N., Toniolo, R., Miranda-Castro, R., de-Los-Santos-Álvarez, N., & Lobo-Castañón, M. J. (2018). Selection of Anti-gluten DNA Aptamers in a Deep Eutectic Solvent. Angewandte Chemie (International ed. in English), 57(39), 12850-12854.

Side projects publications

- Toniolo, R., Dossi, N., Svigelj, R., Pigani, L., Terzi, F., Abollino, O., & Bontempelli, G. (2016). A Deep Eutectic Solvent-based Amperometric Sensor for the Detection of Low Oxygen Contents in Gaseous Atmospheres. Electroanalysis, 28(4), 757-763.

- Toniolo, R., Bortolomeazzi, R., Svigelj, R., Dossi, N., Casella, I. G., Bragato, C., & Daniele, S. (2017). Use of an electrochemical room temperature ionic liquid-based microprobe for measurements in gaseous atmospheres. Sensors and Actuators B: Chemical, 240, 239-247.

- Comuzzo, P., Toniolo, R., Battistutta, F., Lizee, M., Svigelj, R., & Zironi, R. (2017). Oxidative behavior of (+)-catechin in the presence of inactive dry yeasts: a comparison with sulfur dioxide, ascorbic acid and glutathione. Journal of the Science of Food and Agriculture, 97(15), 5158-5167.

- Dossi, N., Petrazzi, S., Toniolo, R., Tubaro, F., Terzi, F., Piccin, E., Svigelj, R., & Bontempelli, G. (2017). Digitally Controlled Procedure for Assembling Fully Drawn Paper-Based Electroanalytical Platforms. Analytical chemistry, *89*(19), 10454-10460.